AD_____

Award Number: W81XWH-10-1-0239

TITLE: The Role of SF2 in Prostate Cancer Progression

PRINCIPAL INVESTIGATOR: Clay Comstock, Ph.D.

CONTRACTING ORGANIZATION: Jefferson Medical College
Philadelphia, PA 19107

REPORT DATE: April 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2011	Annual	1 April 2010 – 31 March 2011

4. TITLE AND SUBTITLE

Role of SF2 in Prostate Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0239

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Clay Comstock

E-Mail: clay.comstock@kimmelcancercenter.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Jefferson Medical College
Philadelphia, PA 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: Prostate cancer (a leading cause of male mortality) development and progression is dependent upon androgen and androgen receptor (AR) signaling. Current therapies target androgen production and/or AR signaling. Evidence suggests that AR in some tumors may escape therapy through mechanisms that likely involve splicing. **Purpose**: To better understand splicing during prostate cancer development or progression. **Scope**: As outlined in the proposal (and recently published) observations suggest that the splicing factor (SF2) may contribute to prostate cancer in part through altered D-cyclin splicing. **Findings to date**: have been on the generation/characterization of constructs to manipulate SF2 levels in normal- and prostate cancer-derived cell model systems. Simultaneous efforts have been made on the development of robust immunohistochemical methods for detection of SF2 in archived prostate tumor specimens. Additional findings, indicate a potential cross-talk between Dcyclins and androgen/AR that may alter splicing/signaling pathways that impact SF2 function.

15. SUBJECT TERMS
SF2, cyclin, androgen receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	32	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Background/Subject: Prostate cancer (PCa) is one of the most prevalent non-cutaneous cancers in men and the second leading cause of mortality after lung cancer (1). PCa is a multi-factorial disease with genetic, hormonal, and environmental components. A critical facet of PCa development and progression is its dependence on androgen signaling as mediated by the androgen receptor (AR) (2). Current therapeutic options range from surgery/radiation for localized disease to deprivation of androgen and/or AR signaling in more aggressive, advanced/metastatic disease. Chemotherapy is not particularly beneficial; however, androgen deprivation therapies (ADT) result in effective disease management for 2-3 years before incurable Castrate-resistant PCa (CRPC) develops (3).

General Purpose: To better understand splicing in PCa and identify novel therapeutic pathways. Especially, as recent evidence suggests isoforms of AR, likely derived through alternative splicing, may contribute significantly to disease progression (4). Unfortunately, little is known concerning the mechanisms, pathways, and/or components of splicing in PCa.

Overall Scope: Previously it been demonstrated that cyclin D1, a key modulator of androgen/AR-dependent transcription and proliferation, is alternatively spliced in PCa (5). Published data have suggested that SF2 and cyclin D1 isoforms have oncogenic functions (6, 7). Based on preliminary data (now published (8)), the current proposal is geared towards determining the consequence of SF2 function and cyclin D1 splicing in the context of PCa.

BODY

The relevant **findings to date (bold)**: are summarized within the overall tasks, aims, and subaims (specific details and projected timelines were provided in the initial Proposal and Statement of Work, respectively). Relevant data that is published and directly pertains to the current Tasks will be summarized and cited or will be provided in reproduction, as indicated, for ease of committee evaluation. Subaims that are to be addressed in subsequent years are indicated and relevant data that is applicable to multiple subaims will also be indicated.

TASK 1

Overview: The overall goal of Task 1 is to essentially determine the impact (Aim A) and relevance (Aim B) of SF2 function and cyclin D1 splicing in the context of PCa.

Aim A: is focused on the identification of PCa cell model systems and their subsequent manipulation of SF2 and cyclin D1 to mimic human disease (Subaim 1). The intended goal is to characterize the pathways and tumorigenic activity of these modified cell lines *in vitro* (Subaim 2) and eventually determine specificity through RNAi technology and inhibitory treatments that alter functional activity (Subaim 3 - to be addressed in subsequent years).

Subaim 1: Generate PCa cells to mimic human disease.

Summary: Preliminary data, in the initial Proposal, from a publically available gene expression database suggests that mRNA for SF2 is elevated as a function of PCa progression.

Strategy: Identify cell model systems that are representative of PCa and are amenable to manipulation of SF2 in order to mimic disease.

Accomplishments:

LNCaP, LAPC4, and VCaP cells are suitable cell model systems for manipulating SF2

As outlined in the initial Proposal, published data in PCa cell model systems have already established the expression levels of cyclin D1 spliced isoforms (5). Therefore, current emphasis has been on characterizing at the protein level which of those cell lines is suitable for transient SF2 manipulation. Accomplishment of this aspect of the proposal is critical for subsequent *in vitro* analyses in Task 1 and generation of stable-isogenic lines for the *in vivo* xenograft studies in Task 2. To this end, immunoblot analysis of SF2 was performed in lysates from LNCaP, LAPC4, and VCaP cells (**Figure 1**). These cell lines are well-characterized PCa cell model systems and are representative of the vast majority of AR-positive PCa. Importantly, the LNCaP cell line, which has been established as having low levels of the spliced isoform of cyclin

D1, demonstrated low levels of SF2 protein making them amenable to over-expression studies required for Subaim 2. In contrast, LAPC4 and VCaP cells demonstrated higher levels of SF2 protein; thus, will be important model systems for future studies that challenge specificity in Subaim 3.

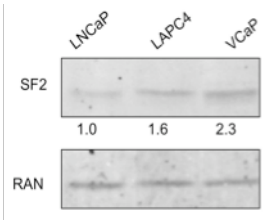

Figure 1. Prostate cancer cell model systems for SF2 manipulation. Immunoblot analysis and quantification, using the LI-COR detection system, of SF2 (upper) relative to the loading control Ran (lower) in prostate cancer (PCa) cell model system lysates. The LNCaP, LAPC4, and VCaP cell lines are highly representative of androgen receptor (AR) positive disease. SF2 levels are adjusted relative to LNCaP cells.

Subaim 2: Characterize tumorigenic activity of PCa cell lines *in vitro*.

Summary: The capacity and/or pathways of SF2 that promote tumor progression are unknown in PCa. While preliminary evidence indicates that SF2 mediates alternative splicing of cyclin D1 it does not rule out the possibility that other pathways may also contribute. Similarly, as cyclin D1 is known to modulate androgen/AR-dependent transcription in PCa cells it is feasible that potential feed-forward and –backward transcriptional mechanisms are invoked that may influence SF2-mediated functions.

Strategy: Develop a robust and transient over-expression system in the LNCaP cell line to study the contributions on gene expression and/or splicing as well as study the tumorigenic potential *in vitro*. Furthermore, establish a stable-isogenic cell line for future *in vivo* studies outlined in Task 2.

Accomplishments:

Expression of SF2-associated factors are altered by transient over-expression of D cyclin

Utilizing the LNCaP cell line, described in Figure 1, transient studies were conducted to study the contributions of elevated cyclin D1 on gene expression. Based on pre-existing methodology and published data, a robust and transient over-expression of cyclin D1 was performed to evaluate the overall transcriptional impact on AR-dependent signaling and these data were recently published (9). Evidence from this study identified gene expression changes in CLK1 (preliminary data reproduced here, as **Figure 2**). Importantly, CLK1 is a known kinase that serves as a critical signaling node to regulate the functional activity of SF2. While preliminary, this observation suggests a novel regulatory loop between D-cyclins and SF2. Furthermore, this data provides valuable information related to dissecting signaling pathways as part of the future specificity studies described in Subaim 3.

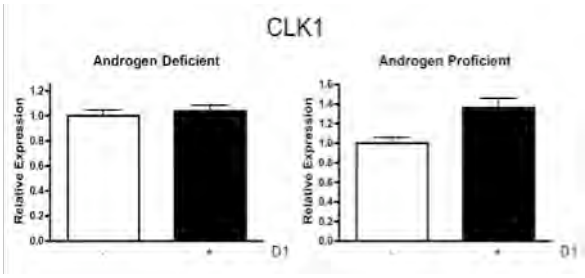

Figure 2. CLK1 (SF2-associated kinase) is altered by D1 cyclin. Gene expression array analysis of CLK1 from LNCaP cells transiently over-expressing D1 (black bars) in the absence (left panel) and presence (right panel) of androgen. Data are reproduced from supplemental data within Comstock, et.al. 2011. *J Biol Chem*. 286(10): 8117.

Elevated SF2 altered cyclin D1 splicing and transcription of SF2-associated genes

SF2 expression studies were initially presented in the Background/Preliminary Data section of the proposal and have now been validated and published (8). These data demonstrated that SF2 mediates alternative splicing of cyclin D1 in PCa cells. Current emphasis has been on the development of a robust and transient over-expression of SF2 in LNCaP cells to identify potential transcriptional and/or splicing alterations in PCa. By representative immunoblot analysis of SF2 (**Figure 3A**), transfection conditions have been established that result in a high-level of SF2 expression. Furthermore, over-expression of SF2 significantly altered the

expression of candidate genes (**Figure 3B**) that are known to be associated with SF2 (e.g., CLK1 and C1qBP) while having minimal impact on genes not known to be associated with SF2 (e.g., KHDRBS3). These data are instrumental in developing unbiased methodologies to accurately identify transcriptional, splicing, and biological changes induced by SF2 over-expression in the LNCaP model system and interpretation of data obtained in Task 2.

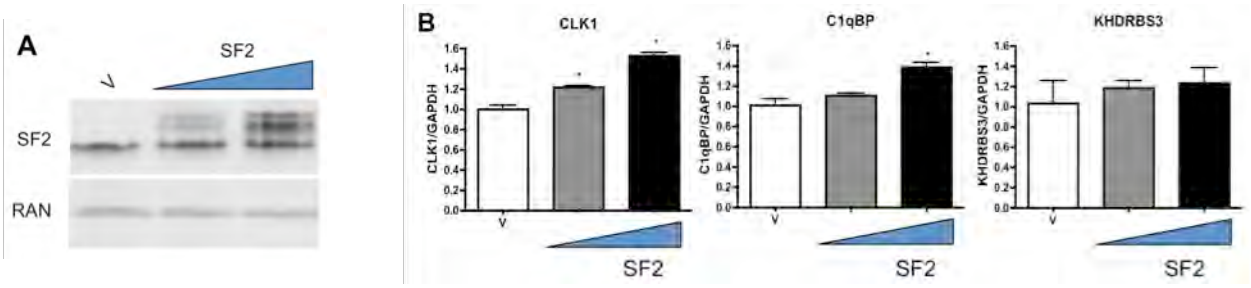

Figure 3. Transiently over-expressed SF2 alters the gene expression of SF2-associated factors. A protocol for robust and transient over-expression of SF2 was developed in order to study the impact on gene expression and splicing. **A**) Representative immunoblot analysis of SF2 in LNCaP cell lysates relative to vector transfected (V, pCGT). SF2 was expressed using two plasmid concentrations of pCGT containing an N-terminal T7-epitope tagged SF2. **B**) Gene expression analysis for factors associated with SF2 function: CLK1 (left) C1qBP (middle); or not associated: KHDRBS3 (right).

Aim B: is focused on determining the relevance of SF2 and cyclin D1 splicing in PCa. Specifically, human prostate specimens with know clinical parameters are to be obtained and archived (Subaim 1). The intent is to stain (Subaim 2) and analyze correlates (Subaim 3 - to be addressed in subsequent years) to determine the relevance to known clinical parameters and/or outcome.

Subaim 1: Obtain and archive clinical prostate specimens.

Summary: Previous analysis was performed using a commercially available PCa tissue microarray that had limited clinical information.

Strategy: Obtain human PCa specimens with known clinical parameters from the university hospital and stain to establish the relevance in PCa and correlate with clinical outcome.

Accomplishments:

Obtained human PCa tumors with adjacent normal (n=30) and metastatic tumors (n=2)

The currently collected specimens will provide ample tissue to perform subsequent correlative analyses. In addition, approximately 500 biopsy specimens are also available with known clinical parameters to establish relevance. Utilizing the staining protocols outlined in Subaim 2, the next step is to identify cohorts of patient specimens to analyze for clinical relevance (Subaim 3).

Subaim 2: Immunohistochemical staining of archived human specimens obtained.

Summary: While it is known that the spliced isoform of cyclin D1 is elevated in human PCa as compared to non-neoplastic prostate and correlates, as described below, with SF2 levels. It is unknown what the association of these proteins is to clinical parameters. Thus, assessment of either of these factors in specimens with known clinical parameters and/or outcome is critical for establishing relevance.

Strategy: To accomplish this objective the strategy is relatively straightforward; stain the collected tumors with available antibodies that have been shown to be specific in prostate tissue. However, based on limitations described in more detail below, the AQUA system (as suggested in the initial Proposal) did not provide the desired mechanistic insight or general compatibility. Therefore, focus has been on optimizing the published antibodies for a method compatible with other published staining protocols (i.e., Ki67) and more amenable to analyzing larger cohorts of specimens.

Accomplishments:

Expression of SF2 and the spliced isoform of cyclin D1 correlate in PCa specimens

Fluorescence-based AQUA analysis using a tissue microarray of high-grade human PCa specimens identified a significant correlation, with glandular resolution, between SF2 and the spliced isoform of cyclin D1 and these data have been recently published. However, the AQUA platform was unable to determine correlations at the cellular level using serial-section stained slides and is not very compatible for direct comparisons with most DAB-based staining protocols available. Furthermore, using this small cohort (n=50) only a trend was discernable between SF2 levels and tumor grade (**Figure 4A**). Thus, current efforts have focused on the development of a cross platform cost-effective immunohistochemical method to screen a larger cohort of PCa specimens to more accurately ascribe relevance (**Figure 4B**) with regard to available clinical parameters and/or outcome. As shown by representative images, the current method provides a dose-dependent SF2 signal primarily in the epithelial compartment and to a lesser extent in the stromal compartment consistent with its function as an important and general splicing factor.

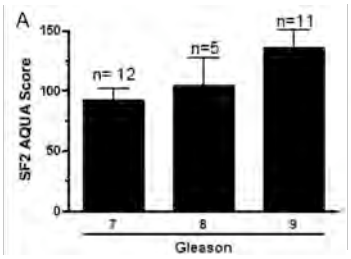

Figure 4. IHC analysis of SF2 in PCa. **A**) F*luorescence-based* SF2 staining in PCa specimens suggesting a trend with Gleason grade. Preliminary data from published IHC staining in Olshavsky, et.al. 2010. *Cancer Res.* 70(10):3975. **B**) DAB-based SF2 staining for a more compatible protocol to cost-effectively screen larger PCa tissue cohorts.

TASK 2

Overview: The general intent of Task 2 is to complement those studies in Task 1 by determination of the *in vivo* consequence of SF2 function and cyclin D1 splicing in the context of PCa development and/or progression (Aim A) and characterization of the response to first line androgen-deprivation therapy (Aim B - to be addressed in subsequent years).

Aim A: is designed to determine the impact of these factors during development/progression of PCa by generating viral constructs (Subaim 1) to genetically modify isolated human or mouse prostate epithelial cell lines (Subaim 2) and perform *in vivo* tissue recombination studies (Subaim 3- to be addressed in subsequent years).

Subaim 1: Generate viral infection constructs.

Summary: Tissue recombination studies, while powerful for mimicking *in vivo* epithelial/stromal interactions are dependent upon the isolation of primary cells or use of transformed but non-tumorigenic cell lines (10). During my training in Owen Witte's laboratory and subsequent communications with Simon Hayward (both experts in the tissue recombination field) it became evident that recombination studies with transfected, prostate-epithelial primary cells or lines is difficult due to the low transfection efficiency. Therefore, it is critical to develop viral constructs to allow production of the desired proteins with high-efficiency.

Strategy: The development of viral constructs is centered on the Gateway cloning method (Invitrogen). The cloning strategy involves initial cloning the gene of interest into a viral entry vector and sequencing. Subsequently, the entry vector is recombined with a viral destination vector that is suitable for expression screening analysis or viral production.

Accomplishments:

Generation of viral constructs for SF2 expression

Utilizing the T7-SF2 vector (used for over-expression studies as shown in Task 1): oligonucleotides were designed to amplify T7-SF2 and the subsequent fragments were cloned into the pENTR vector that contains elements needed for recombination with the viral production vectors. Following verification by PCR (**Figure 5A,** representative clone shown) and

sequencing (data not shown) the pENTR/T7-SF2 vector was recombined with the pDEST vector and clones were obtained with the proper size and orientation as determined by restriction endonuclease digestion (**Figure 5B,** representative clone shown). Currently, the pDEST/T7-SF2 clones are being evaluated for correct expression. Once expression is verified virus will be generated and tested for infection efficiency and protein expression.

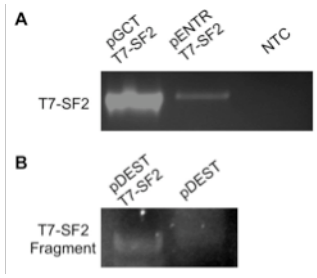

Figure 5. Generation of viral constructs for SF2 transduction. Generation of viral constructs is based on the Gateway system (Invitrogen). Viral constructs are critical for transducing genes-of-interest into hard to transfect cells like primary and/or spontaneously immortalized normal prostate cells. **A**) PCR amplified: T7-SF2 from the pCGT/T7-SF2 (described in Figure 3) and cloned T7-SF2 from the pENTR/T7-SF2 vector containing elements for recombination into the pDEST viral vector. NTC=non-template control. **B**) Restriction digest of pDEST/T7-SF2 and pDEST parental vector demonstrating a fragment of correct size and orientation.

Subaim 2: Isolate/infect normal epithelium to generate genetically modified lines.

Summary: The ability to generate successful tissue recombinants *in vivo* with discernable outcomes requires that a large percentage of epithelial cells contain the desired protein of interest. Thus, the generation of viral constructs, as outlined above in Subaim 1, it is critical. Equally important is the ability to use normal-derived prostate lines and/or isolate primary prostate epithelial cells from mouse or human tissues.

Strategy: Two approaches are currently under consideration to identify epithelial populations amenable to modification for recombination studies. First, recently published data has demonstrated the isolation and characterization of epithelial cells derived from normal and benign human prostate tissue that form tissue recombinants *in vivo*. Second, and somewhat more challenging, is the isolation of embryonic mouse prostate epithelial cells that are traditionally used to form tissue recombinants. Current efforts are underway, with regard to the later approach, to isolate pure populations of mouse epithelial cells.

Accomplishments:

SF2 is expressed in normal- and benign-derived human prostate epithelial cell lines

Human epithelial cell lines derived from normal and benign prostate tissue were obtained from Simon Hayward (11). Importantly, these cells are spontaneously immortalized; thus, eliminating any potential contribution of typical immortalizing factors. Furthermore, as these cells are derived from human specimens they should be more equivalent to human primary cells as compared to those derived from mouse. In addition, the established nature of these lines should provide a solid foundation for subsequent modification and more consistent tissue recombinants. As shown by representative immunoblot (**Figure 6**), both the NHPrE1 (normal-derived) and BHPrE1 (benign-derived) cell model systems express SF2 protein. Importantly, those cells derived from normal, as compared to the benign, prostate tissue appear to express less SF2 protein. Current efforts to transiently over-express T7-SF2 have not been very successful due to low transfection efficiency. Thus, completion of this step will likely require the use of viral infection as described in Subaim 1.

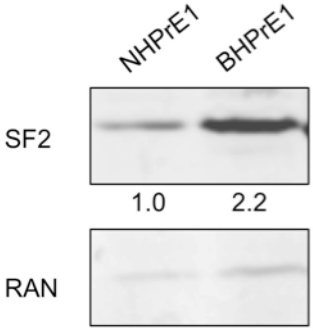

Figure 6. SF2 in normal and benign human prostate epithelial cells. Immunoblot analysis and quantification, using the LI-COR detection system, of SF2 (upper) relative to the loading control Ran (lower) in lysates from spontaneously immortalized normal (NHPrE1) and benign (BHPrE1) human prostate epithelial cell lines obtained from Simon Hayward. SF2 levels are adjusted relative to NHPrE1 cells. Currently, these cells are as close to human primary cell isolates, are amenable to tissue recombination studies proposed in Task 2, and should provide a consistent foundation to manipulate using the constructs described in Figure 5.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 - Impact of SF2/cyclin D1 splicing in PCa cells (Aim A) and tumors (Aim B).

•Identified multiple representative PCa cell model systems to manipulate SF2 and determine the impact in PCa cells (**Figure 1**).

•Manipulated D-cyclins in the LNCaP cell model system (published (9)) and identified CLK1, a known SF2-associated kinase, as a potential signaling node in PCa (**Figure 2**).

•Validated that SF2 expression promotes cyclin D1 splicing in PCa cells (published (8)) and characterized a robust and transient over-expression of SF2 results in transcriptional changes in genes associated with SF2 (**Figure 3**).

•Identified a positive correlation (using the AQUA platform) between SF2 and the spliced isoform of cyclin D1 in PCa specimens (published (8)). Due to a small cohort size only a trend was observed for SF2 and Gleason grade; thus, a more cost-effective immunohistochemical method has been developed to screen a larger cohorts of specimens (**Figure 4**).

Task 2 - Impact of SF2/cyclin D1 splicing in recombination (Aim A) and therapy (Aim B).

•Generated SF2 constructs for viral infection of isolated prostate epithelial cells in order to determine the *in vivo* impact on tumor initiation and splicing (**Figure 5**).

•Characterized SF2 levels in spontaneously immortalized cell lines derived from normal and benign human prostate tissue to determine the impact in a mouse tissue recombination model of prostate development (**Figure 6**).

REPORTABLE OUTCOMES

Manuscripts:

1. Olshavsky, **Comstock** et.al.; 2010. Identification of ASF/SF2 as a critical, allele-specific effector of the cyclin D1b oncogene. *Cancer Res*. May 15; 70(10):3975-84.
2. **Comstock,** Augello et.al.; 2011. Cyclin D1 is a selective modifier of androgen-dependent signaling and androgen receptor function. *J Biol Chem*. Mar 11; 286(10):8117-27.

Abstracts/Presentations:

1. **Comstock** et. al. Keystone Symposia on Nuclear Receptors. Keystone, CO. March 2010 Poster Presentation.

Patents: None

Degrees obtained: N/A

Development of cell lines: In progress

Informatics: None developed

Funding: None applied for

Employment/Research opportunities: None applied for

CONCLUSIONS

Typically, early-stage prostate cancer (PCa) is effectively treated with surgery and/or radiation therapy. However, treatment of more advanced PCa, through traditional modalities that involve androgen deprivation, remains a significant challenge as the disease inevitably transitions into incurable castrate-resistant PCa (CRPC). Therefore, much of the current investigation in the field has centered on the mechanisms that result in CRPC disease. Pertinent to the proposal at hand is a recent development that one CRPC mechanism is alternate splicing of the androgen receptor (AR) that relays androgenic signaling critical for normal prostate function and has been

demonstrated to be a key driver of PCa progression. Unfortunately, little information is known about the components, signaling pathways, and mechanisms of splicing in PCa. Previous work has demonstrated that a key regulator (i.e., cyclin D1) of AR signaling and proliferation is alternatively spliced in PCa. Preliminary data, outlined in the original proposal, suggested that a well-known splicing factor (i.e., SF2) regulates cyclin D1 splicing. Importantly, these data have been confirmed and recently published; wherein, SF2 levels correlated with the spliced form of cyclin D1 and splicing was dependent upon a polymorphism. As outlined in the proposal, it remains to be determined: 1) what is the overall impact and clinical relevance of SF2, cyclin D1, and splicing in PCa cell model systems (Task 1A) and human specimens (Task 1B); 2) what is the consequence of these factors to tumor initiation and progression (Task 2A); and 3) how do these factors contribute to first-line, androgen-deprivation therapy (Task 2B).

Progress has been made with regard to the Statement of Work. First, cell model systems that are highly representative of PCa have been characterized for subsequent studies involving over-expression (Task 1A, Subaim 1/2) and knockdown/inhibitors (Task 1A, Subaim 3). Second, additional preliminary data from our published observations suggests that a potential regulatory loop between D-cyclins and SF2 may exist that should be beneficial for interpretation of outcomes (Tasks 1/2). Third, preliminary data derived from our initial published observations indicate a trend between SF2 and Gleason grade. However, for reasons stated below (Alternatives section) a more cost-effective and compatible immunohistochemical protocol has been developed to address clinical relevance in larger cohorts of archived biopsy and collected tumor specimens (Task 1B). Finally, viral constructs have been made and prostate epithelial lines characterized; which are critical for studies involving *in vivo* analyses (Task 2A/B).

So What: The current progress is in line with the intended goals of the proposal and, provided no unforeseen complications arise, should provide the foundation to discern the impact and consequence of these factors in PCa. Furthermore, completion of these tasks will provide essential information with regard to splicing, an important and emerging field of prostate biology, and will shed light on the potential ramifications of these factors on first-line therapies.

Alternatives: Based on the preliminary immunohistochemical data, it was proposed to use the fluorescence-based AQUA platform to identify clinical relevance in archived biopsy and collected tumor specimens. At the time of the proposal, it was thought that the AQUA system would provide the opportunity to observe mechanistic detail down to individual cells; however, analysis could only make determinations down to individual glands. In addition, analysis of each tumor spot on a tissue microarray (published data, n~50) is rather costly and associations with Gleason grade (preliminary data herein) indicated a trend suggesting that additional tumors would be required to define significance. Furthermore, the continuous fluorescence-based signal is not particularly conducive to direct comparisons with traditional DAB-based signals and quantification. Therefore, the antibodies have been optimized for DAB-based staining that will allow a more direct comparison with other traditional staining protocols (e.g., Ki67) in order to identify associations with clinical parameters.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E Cancer statistics, 2010. CA Cancer J Clin 60:277-300
2. Taplin ME 2007 Drug insight: role of the androgen receptor in the development and progression of prostate cancer. Nat Clin Pract Oncol 4:236-244
3. Ryan CJ, Smith A, Lal P, Satagopan J, Reuter V, Scardino P, Gerald W, Scher HI 2006 Persistent prostate-specific antigen expression after neoadjuvant androgen depletion: an early predictor of relapse or incomplete androgen suppression. Urology 68:834-839
4. Shiota M, Yokomizo A, Fujimoto N, Naito S Androgen Receptor Cofactors in Prostate Cancer: Potential Therapeutic Targets of Castration-Resistant Prostate Cancer. Curr Cancer Drug Targets
5. Comstock CE, Augello MA, Benito RP, Karch J, Tran TH, Utama FE, Tindall EA, Wang Y, Burd CJ, Groh EM, Hoang HN, Giles GG, Severi G, Hayes VM, Henderson BE, Le Marchand L, Kolonel LN, Haiman CA, Baffa R, Gomella LG, Knudsen ES, Rui H,

Henshall SM, Sutherland RL, Knudsen KE 2009 Cyclin D1 splice variants: polymorphism, risk, and isoform-specific regulation in prostate cancer. Clin Cancer Res 15:5338-5349

6. Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR 2007 The gene encoding the splicing factor SF2/ASF is a proto-oncogene. Nat Struct Mol Biol 14:185-193
7. Sanchez G, Delattre O, Auboeuf D, Dutertre M 2008 Coupled alteration of transcription and splicing by a single oncogene: boosting the effect on cyclin D1 activity. Cell Cycle 7:2299-2305
8. Olshavsky NA, Comstock CE, Schiewer MJ, Augello MA, Hyslop T, Sette C, Zhang J, Parysek LM, Knudsen KE Identification of ASF/SF2 as a critical, allele-specific effector of the cyclin D1b oncogene. Cancer Res 70:3975-3984
9. Comstock CE, Augello MA, Schiewer MJ, Karch J, Burd CJ, Ertel A, Knudsen ES, Jessen WJ, Aronow BJ, Knudsen KE Cyclin D1 is a selective modifier of androgen-dependent signaling and androgen receptor function. J Biol Chem 286:8117-8127
10. Strand DW, Franco OE, Basanta D, Anderson AR, Hayward SW Perspectives on tissue interactions in development and disease. Curr Mol Med 10:95-112
11. Jiang M, Strand DW, Fernandez S, He Y, Yi Y, Birbach A, Qiu Q, Schmid J, Tang DG, Hayward SW Functional remodeling of benign human prostatic tissues in vivo by spontaneously immortalized progenitor and intermediate cells. Stem Cells 28:344-356

Cancer Research

Identification of ASF/SF2 as a Critical, Allele-Specific Effector of the Cyclin D1b Oncogene

Nicholas A. Olshavsky[1,3], Clay E.S. Comstock[2,3], Matthew J. Schiewer[2,3], Michael A. Augello[2,3], Terry Hyslop[2,5], Claudio Sette[6], Jinsong Zhang[1], Linda M. Parysek[1], and Karen E. Knudsen[2,3,4]

Abstract

The cyclin D1b oncogene arises from alternative splicing of the *CCND1* transcript, and harbors markedly enhanced oncogenic functions not shared by full-length cyclin D1 (cyclin D1a). Recent studies showed that cyclin D1b is selectively induced in a subset of tissues as a function of tumorigenesis; however, the underlying mechanism(s) that control tumor-specific cyclin D1b induction remain unsolved. Here, we identify the RNA-binding protein ASF/SF2 as a critical, allele-specific, disease-relevant effector of cyclin D1b production. Initially, it was observed that SF2 associates with cyclin D1b mRNA (*transcript-b*) in minigene analyses and with endogenous transcript in prostate cancer (PCa) cells. SF2 association was altered by the *CCND1* G/A870 polymorphism, which resides in the splice donor site controlling *transcript-b* production. This finding was significant, as the A870 allele promotes cyclin D1b in benign prostate tissue, but in primary PCa, cyclin D1b production is independent of A870 status. Data herein provide a basis for this disparity, as tumor-associated induction of SF2 predominantly results in binding to and accumulation of G870-derived *transcript-b*. Finally, the relevance of SF2 function was established, as SF2 strongly correlated with cyclin D1b (but not cyclin D1a) in human PCa. Together, these studies identify a novel mechanism by which cyclin D1b is induced in cancer, and reveal significant evidence of a factor that cooperates with a risk-associated polymorphism to alter cyclin D1 isoform production. Identification of SF2 as a disease-relevant effector of cyclin D1b provides a basis for future studies designed to suppress the oncogenic alternative splicing event. *Cancer Res; 70(10); 3975–84.* ©2010 AACR.

Introduction

The cyclin D1b variant, produced via alternative splicing of the *CCND1* transcript (1, 2), is a potent oncogene that harbors distinct functions from full-length cyclin D1 (cyclin D1a; refs. 3–5). Unlike cyclin D1a, cyclin D1b independently confers cellular transformation (4, 5). In addition, only the cyclin D1b isoform has the capacity to promote anchorage-independent growth and cell invasiveness (6). Further confirmation of novel oncogenic capabilities was identified in mouse models, wherein animals expressing human cyclin D1b under the bovine K5 promoter showed increased papilloma multiplicity (3). Given the enhanced oncogenic function of cyclin D1b, it is imperative to define the mechanisms that regulate cyclin D1b production in systems of clinical relevance.

The alternative splicing event that produces the cyclin D1b transcript (referred to as *transcript-b*) arises from failure to splice at the *CCND1* exon 4–intron 4 boundary. Due to intronic transcriptional termination, cyclin D1b lacks exon 5–encoded sequences and contains a novel COOH-terminal domain of unknown function (1, 2). Previous studies showed that *transcript-b/transcript-a* ratios are enhanced in selected tumor types, thus providing evidence that the alternative splicing event may be altered as a function of tumorigenesis or tumor progression (6–10). Lending support to this posit, recent analyses of a large cohort of prostate cancer (PCa) specimens revealed that cyclin D1b (but not cyclin D1a) is induced in PCa as compared with nonneoplastic tissue (11). These findings were of interest, as cyclin D1b has specialized functions in this tumor type that are hypothesized to promote tumor progression (7).

Despite the compelling evidence identifying cyclin D1b as a potent, novel oncogene, the tumor-associated factor(s) that promote the alternative splicing event remain poorly defined. It has long been suggested that a polymorphism within the exon 4 splice donor site (G/A870) might contribute to *transcript-b* production, wherein the A allele was suggested to favor the alternative splicing event (1, 12, 13). Recent analysis using minigenes supported this contention,

Authors' Affiliations: [1]Department of Cancer and Cell Biology, University of Cincinnati, Cincinnati, Ohio; [2]Kimmel Cancer Center, Departments of [3]Cancer Biology, [4]Urology, and [5]Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, Pennsylvania; and [6]Department of Public Health and Cell Biology, Section of Anatomy, University of Rome Tor Vergata, and Centro Europeo di Ricerca sul Cervello (CERC), Fondazione Santa Lucia, Rome, Italy

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Karen E. Knudsen, Thomas Jefferson University/Kimmel Cancer Center, 233 South 10th Street, Bluemle Building, Room 1008, Philadelphia, PA 19107. Phone: 215-503-8574; Fax: 215-923-4498; E-mail: karen.knudsen@kimmelcancercenter.org.

doi: 10.1158/0008-5472.CAN-09-3468

and analysis of nonneoplastic prostate tissue showed that the presence of the A allele predicted for higher *transcript-b* production; however, the effect of the A allele was lost in tumor tissue, thus indicating that tumor-associated factor(s) likely bypass or modify the effect of the G/A870 polymorphism with regard to *transcript-b* production (11). Here, the present study identifies the SF2 (also known as ASF or SRp30a) RNA binding protein as a critical, allele-selective factor that associates directly with *transcript-b* and modulates cyclin D1b production in model systems of cancer relevance. Importantly, analyses of tumor tissue further support a model wherein tumor-associated elevation of SF2 specifically enhances cyclin D1b expression in human disease. Together, these findings provide a mechanism by which *CCND1* alternative splicing is controlled in tumorigenesis, and identify SF2 as a critical regulator of cyclin D1b oncogene production.

Materials and Methods

Cell culture, transfections, generation of stables. LNCaP, C33A, and LAPC4 cell lines were obtained, cultured, and transfected as previously described (7, 14–17). The DT40-ASF cell line was a generous gift from James Manley (Cell and Molecular Biology, Columbia University, New York, NY) and maintained as previously described (18). DT40-ASF cells were transfected using the Amaxa Nucleofector protocol. C33A stable cell lines were generated by transfecting the indicated expression constructs encoding empty vector, or individual cyclin D1 minigenes and selected with 400 μg/mL of G418 (MP Biomedicals). Clonal isolates were screened for expression by immunoblot. Isolates used herein are denoted as C33A-Vec, C33A-G1 (containing the G870 allele minigene), and C33A-A1 (containing the A870 allele minigene).

Plasmids. The pCGT7, pCGT7-ASF/SF2, and pCGT7-SRp40 expression constructs were generous gifts from Adrian Krainer (Biological Sciences, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and have been previously described (19). pCEP4-ASF/SF2 was a generous gift from Woan Yuh-Tarn (Cell Biology and Signal Transduction, Institute of Biomedical Sciences, Taipei, Taiwan) and was previously described (20). The cyclin D1 minigenes were previously described (11).

Immunoblotting. Cells were harvested, lysed in NETN, and subjected to SDS-PAGE and immunoblotting as previously described (14, 15). Immunoblots were performed using antisera against ASF/SF2 (Invitrogen), β-tubulin (Sigma), cyclin D1a (Neomarker, Ab3), cyclin D1b (7), glyceraldehyde-3-phosphate dehydrogenase (Invitrogen), or T7 (Novagen), as indicated.

RNA isolation, PCR, PCR-restriction fragment length polymorphism, and real-time PCR. RNA was isolated using TRIzol according to the protocols of the manufacturer. PCR analyses and primer sequences for *transcript-a, transcript-b*, and glyceraldehyde-3-phosphate dehydrogenase were previously described (7, 15, 21). The PCR-restriction fragment length polymorphism (PCR-RFLP) was performed as previously described (7, 22); briefly, RNA immunoprecipitation (RIP)–recovered products were subjected to a second amplification using primers designed for RFLP analyses. The product was then digested and visualized after electrophoresis by ethidium bromide. Real-time PCR for *transcript-b* was performed using the Syber Green protocol from Applied Biosystems and a 7900F Real-time PCR machine.

Immunohistochemistry and AQUA system analysis. Immunohistochemistry and AQUA analyses were performed as previously described (11) using a human prostate cancer array (SuperBio Chips Labs) and antisera indicated above. Comparison of relative SF2 levels from microarrays were analyzed using one-way ANOVA. Correlations of SF2 with cyclin D1a and D1b AQUA expression levels was completed using Spearman correlation coefficients, with Bonferroni correction of P values for multiple comparisons.

RNA immunoprecipitation. RIPs were performed as previously described (23). Briefly, cells (LNCaP, LAPC4, C33A, and the C33A-derived stable cell lines) were harvested and nuclear extracts prepared by resuspending in isotonic buffer. After a 7-minute incubation on ice, samples were centrifuged at 700 × g for 7 minutes and nuclei were isolated, resuspended in buffer supplemented with 90 mmol/L of NaCl and 0.5% Triton X-100, and sonicated. After centrifugation at 5,000 × g for 15 minutes, nuclear extracts were precleared for 1 hour and immunoprecipitated with ASF/SF2 antibody or mouse IgG antibody. The antibody-antigen complex was precipitated by the addition of protein G-Sepharose beads (Invitrogen) for 3 hours at 4°C with rotation. Beads were washed thrice with lysis buffer and an aliquot was eluted in SDS sample buffer for immunoblot analysis. The remaining beads were incubated with lysis buffer in the presence of (RNase free) DNase (Ambion) for 15 minutes at 37°C and washed thrice with lysis buffer before incubation with 50 μg of proteinase K (Roche) for 15 minutes at 37°C. Coprecipitated RNA was then extracted by the TRIzol procedure and used for reverse transcription-PCR analyses.

Results

G/A870 polymorphism alters cyclin D1b production and SF2 binding. Given the enhanced oncogenic function of the cyclin D1b variant (3–5), and the established observation that cyclin D1b levels are enhanced as a function of prostate tumorigenesis (7, 11), it is imperative to discern the mechanism(s) underpinning the alternative splicing event. As was recently reported, the *CCND1* G/A870 polymorphism plays a context-specific role in the alternative splicing event, wherein the A allele favors cyclin D1b production in minigene expression studies in which the alleles were individually examined (11). These studies were validated here, wherein minigenes harboring either the G or A870 allele (Fig. 1A) were independently introduced by stable transfection into C33A cells, which have been previously shown to harbor low to undetectable levels of endogenous cyclin D1 isoforms (24). Introduction of the G allele minigene (stable line C33A-G1), resulted in detectable expression of both *CCND1* transcripts (*a* and *b*), but a preference towards *transcript-a* and cyclin D1a protein was observed (lane 2). *Transcript-b* levels were comparatively increased in the presence of the A allele minigene (lane 3), consistent with previous studies in which the minigenes were individually analyzed in parallel (11).

Subsequent analyses of individual minigene studies in spontaneously immortalized CV1 cells or viral oncoprotein immortalized RWPE-1 prostate epithelial cells (25, 26) were also carried out. Similar findings were observed in both nontumorigenic model systems, in which the A allele predisposed to *transcript-b* and cyclin D1b production (Supplementary Fig. S1). These studies further support the premise that in isolated comparisons, the G/A870 polymorphism influences *CCND1* alternative splicing.

As G/A870 lies within the splice donor site, it was reasoned that the polymorphism might influence RNA binding protein recognition or activity. The splicing factor binding resource ESEfinder3.1 (27, 28) was therefore used to predict distinctions in the profile of associated splicing factors between the G and A-870 allele splice donor sites. As shown, binding of SF2 was suggested to be altered in both position and strength by changes in the polymorphic site (Fig. 1C). To initially assess whether SF2 could play a role in *CCND1* alternative splicing, RIP was performed using the same stable cell lines described in Fig. 1B. Using standard techniques, RNA associated with either SF2-specific or control antisera were isolated, reverse transcribed, and detected by PCR amplification. As illustrated in Fig. 1D (top), *transcript-b* was only weakly detected in control cells and immunoprecipitates thereof (lanes 1–3). In cells containing the G allele minigene (C33A-G1), SF2 readily associated with *transcript-b*, providing the first evidence that SF2 associates with this mRNA species (lanes 4–6). SF2 association with *transcript-b* was reduced in cells containing the A allele minigene (lanes 7–9), as was confirmed by quantitative reverse transcription-PCR (Fig. 1D, bottom). For these analyses, signals observed in C33A-Vec cells were set to "1" for ease of comparison. The finding that SF2 binding is influenced by the polymorphism was subsequently confirmed in additional stable isolates of minigene integration (Supplementary Fig. S2A) as well as through transient analyses (B). Together, these data strongly indicate, for the first time, that the G/A870 polymorphism influences recognition by the SF2 splicing factor.

SF2 specifically correlates with cyclin D1b in prostate cancer. The observation that the G/A870 polymorphism may alter SF2 association with *transcript-b* was of interest, given reports that cyclin D1b, not cyclin D1a, is induced as a function of prostate tumorigenesis (7, 11), and that cyclin D1b has oncogenic activity (3–5). Intriguingly, analyses of gene microarray data from human PCa showed that SF2 expression increases with tumor progression (Fig. 2A; refs.

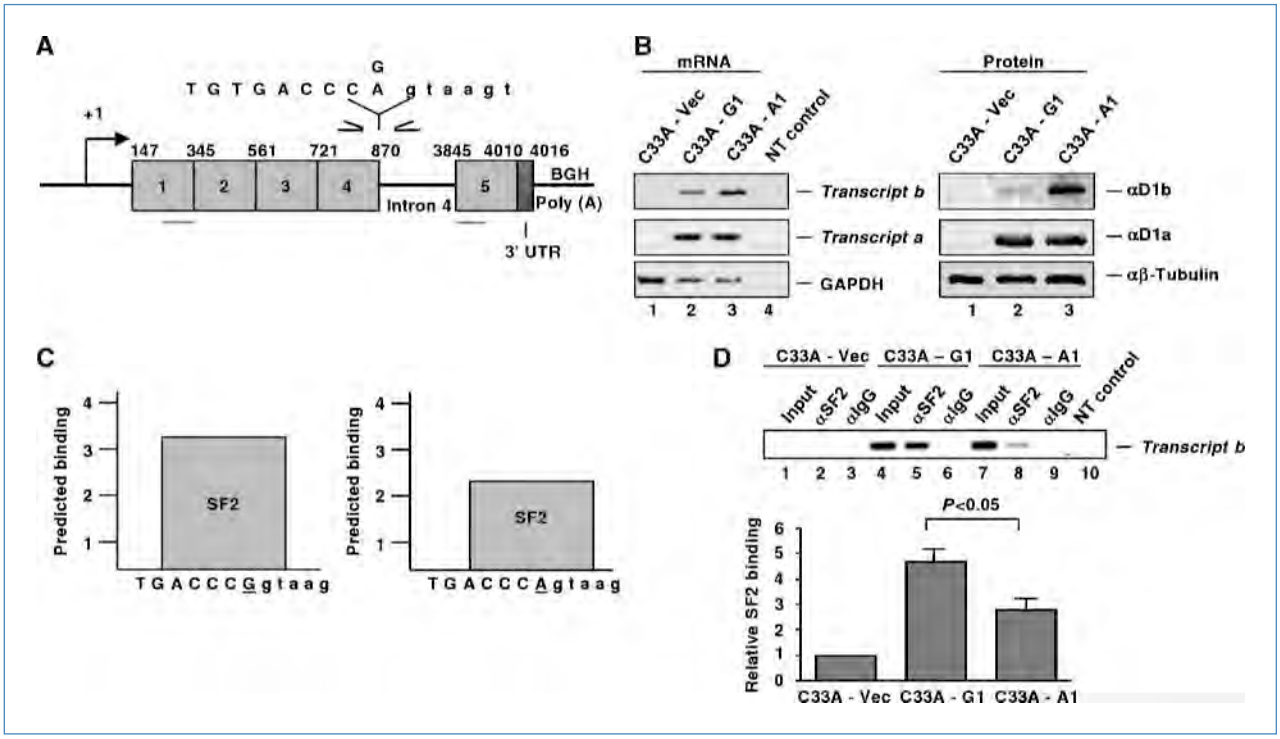

Figure 1. SF2 associates with *transcript-b* and shows allele preference in minigene analyses. A, schematic of the pcDNA3.1-cyclin D1-intron 4 minigenes. Primers used to amplify *transcript-a* (bars; exon 1 and exon 5) and *transcript-b* (arrows; exon 4 and intron 4) are indicated. Clonal isolates C33A-Vec, C33A-G1, and C33A-A1 are as described in the Materials and Methods. B, representative mRNA (left) and protein (right) analyses of cell lines engineered to individually express the G870- or A870-containing minigenes. Nontemplate (NT) is the negative control. C, analyses of predicted SF2 binding to the *CCND1* splice donor site as determined using the ESEfinder3.1 resource. Exonic (upper case) and intronic (lower case) sequence is shown, and the G/A870 polymorphic site underlined. D, C33A-derived stable cell lines in B were subjected to a RIP. Representative reverse transcription-PCR reaction of input and SF2 or IgG-associated transcript is shown (top), and quantification by qPCR of at least three independent experiments provided (bottom). Statistical significance ($P < 0.05$) was determined by ANOVA.

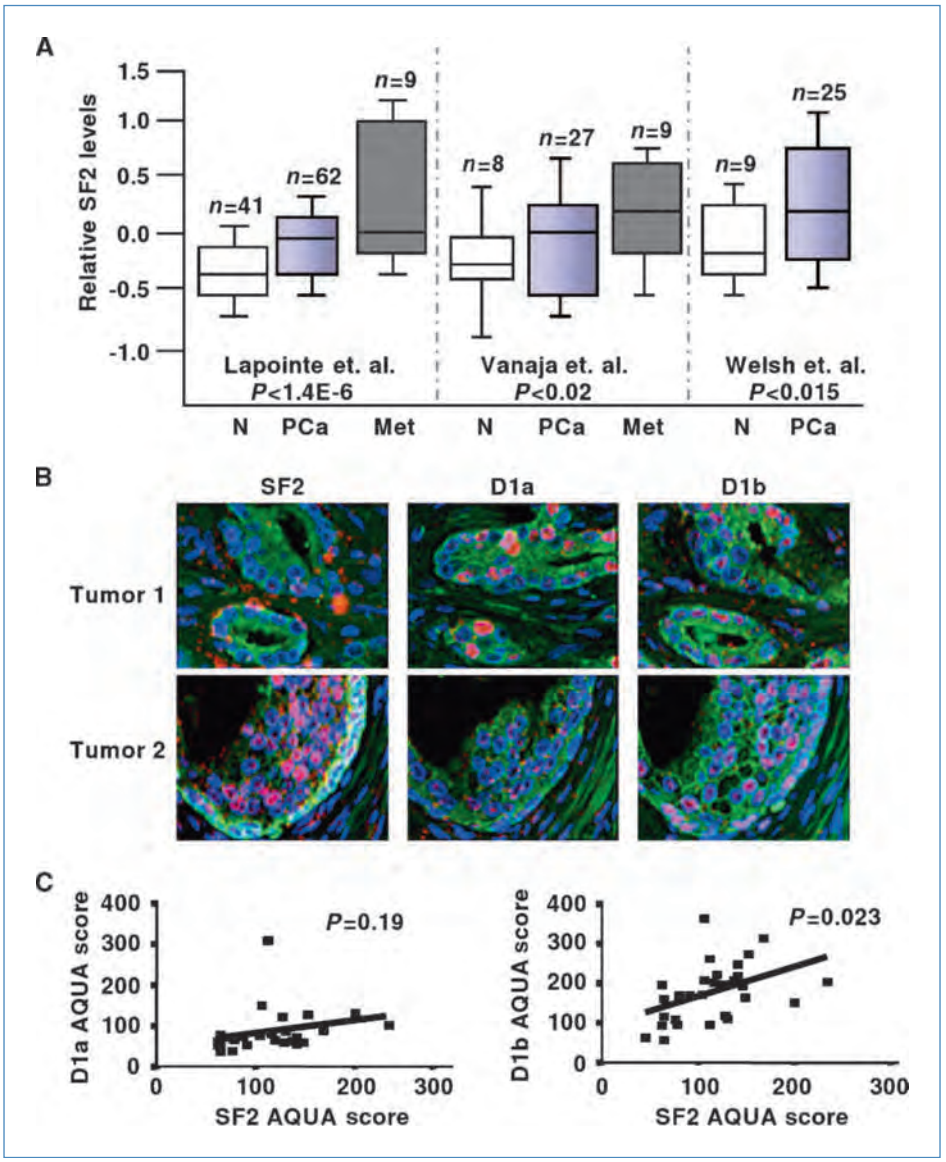

Figure 2. SF2 is enhanced in PCa and positively correlates with cyclin D1b. A, analyses of independent microarrays were determined using the Oncomine resource. Relative SF2 expression in nonneoplastic (N), primary PCa (PCa), and metastatic PCa (Met) are shown and *P* values given. B, representative immunostaining of tissue microarrays using the AQUA platform. As previously described, cells of epithelial origin were identified using a pan-cytokeratin antisera (green), nuclei were stained with DAPI (blue), and cyclin D1 isoforms detected in serial sections using well-characterized, isoform-specific antisera (red). C, quantification of all samples analyzed (n = 49) across each core was used to determine the relevance of SF2 for cyclin D1a (left) and cyclin D1b (right) levels. *P* values were determined via Spearman rank correlation.

29–31). This observation was further validated upon screening a panel of PCa cell lines, wherein it was revealed that SF2 expression is lowest in nontransformed RWPE-1, and as compared with cells derived from primary and distant metastases of prostate cancer (Supplementary Fig. S3). Among the cancer cell lines representative of hormone therapy–sensitive disease, SF2 was relatively low in cells known to express lower levels of cyclin D1b (LNCaP) and higher in cells of this subtype known to express high levels of cyclin D1 (LAPC4; refs. 7, 11). For the androgen receptor–negative cells, unexpectedly high levels of expression were observed in PC3 cells, which exhibit the most aggressive phenotype *in vivo* with regard to metastases (32). To probe the effect of SF2 on cyclin D1b production in human tumors, comparative analyses of nuclear SF2 and cyclin D1 (a and b isoforms) expression was objectively analyzed using serial sections of PCa specimens and quantitative AQUA analyses. Representative images of tumors scoring with low SF2 (Fig. 2B, top) or high SF2 (bottom) are shown. Signals were quantified across each specimen within the nuclear compartment (DAPI positive, blue) of epithelially derived carcinoma cells (cytokeratin positive, green), and resultant data are plotted in Fig. 2C. As shown, tumors expressing low SF2 exhibited low cyclin D1b expression; conversely, tumors with high SF2 scored high for cyclin D1b. Quantification of all tumors examined revealed a significant correlation between SF2 and cyclin D1b (P = 0.023), but not cyclin D1a (P = 0.19). Coexpression of cyclin D1b and SF2, in serial sections, was observed in glandular epithelial cells (data not shown). Together, these data show that SF2 expression is strongly correlated with only the cyclin D1b isoform in human disease.

SF2 modulates cyclin D1b production in multiple model systems. Because SF2 predicted for high cyclin D1b in PCa, the effect of SF2 on cyclin D1b production was examined in model systems of disease relevance. Initially, LNCaP cells, which express low endogenous levels of cyclin D1b, were used (7). Epitope-tagged SR proteins (SRp40 or SF2) were individually introduced (Supplementary Fig. S4) by transient assays with high transfection efficiency (Supplementary Fig. S5), and the effect on *CCND1* mRNA isoform expression determined (Fig. 3A). Consistent with previous results, these cells express low levels of endogenous *transcript-b* (lane 1), and SRp40 failed to elicit changes in either *CCND1* transcript (lane 2). Significantly, SF2 promoted a dramatic increase in *transcript-b* expression without altering *transcript-a* (lane 3). Quantification revealed a ~2.7-fold increase in *transcript-b* following SF2 introduction (right), thus providing the first functional evidence for SF2-mediated regulation of cyclin D1b. Increased expression of SF2 in LNCaP cells also resulted in an increase in cyclin D1b protein expression (Supplementary Fig. S4). Unfortunately, depletion of endogenous SF2 in PCa models using short interfering RNA were unsuccessful (data not shown), consistent with previous observations that knockdown of SF2 induces cell death (33). As such, a previously developed DT40-ASF/SF2 cell line was used (DT40-ASF), in which endogenous SF2 was replaced with a tet-repressible SF2 construct (Fig. 3B; ref. 18). In this system, SF2 expression was undetectable ~24 hours following the addition of doxycycline. Previous reports indicated that 48 hours post-knockdown, the DT40-ASF cell line begins to undergo apoptosis (33). Thus, experiments analyzed end points after 24 hours, whereupon no loss of cell viability was noted (data not shown). As expected, no detectable cyclin D1 isoforms were recognized (Fig. 3B, left, lanes 1 and 2). Stable introduction of the G870 minigene revealed that although both isoforms were produced (left, lanes 4 and 5), suppression of endogenous SF2 (left, lane 5) diminished cyclin D1b (but not cyclin D1a) production. Similar results were observed at the level of the transcripts following depletion of SF2 (Fig. 3B, right). Collectively, these results identify SF2 as an effector of cyclin D1b production in multiple model systems.

Endogenous SF2 associates with both alleles in PCa cells. As it was observed that elevated SF2 induces cyclin D1b in PCa (Fig. 2), and that SF2 differentially binds the transcript dependent on G/A870 (Fig. 1), the effect of the polymorphism on SF2 association was investigated in PCa cells. First, LAPC4 cells were used, which are homozygous for the A870 allele (7). Consistent with the individual minigene analyses in Fig. 1, endogenous SF2 associates with the endogenous *transcript-b* in these cells (Fig. 4A). RFLP analyses of the SF2-associated transcript was also performed (Fig. 4B). These observations show that in PCa, SF2 can associate with *transcript-b* generated by the A870 allele; however, as individual minigene analyses in Fig. 1 indicated a preference for SF2 to associate with *transcript-b* from the G870 allele (when compared in isolation), the most critical analyses were generated in LNCaP cells, which are heterozygous for the polymorphism (shown as genotype control in Fig. 4B). This model system afforded the first opportunity to dissect the effect of SF2 on G870-derived versus A870-derived transcripts in the same model system. As expected, SF2 was associated with total *transcript-b* in this model system (Fig. 4C). Using the SF2-bound and recovered transcript, two key determinations were made. First, RFLP analyses of the total transcript (Fig. 4D, lane 1) revealed that ~61% of the endogenous *transcript-b* was derived from the G allele and 39% from the A allele, thus indicating that in the endogenous setting, both alleles significantly contribute to *transcript-b* production. Intriguingly, RFLP analyses and

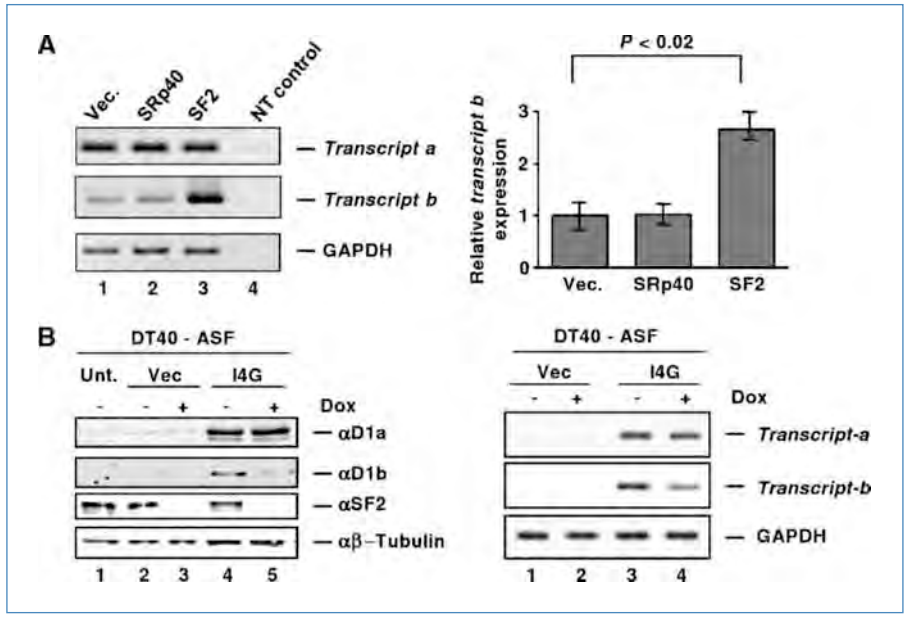

Figure 3. Cyclin D1b production was induced by SF2 in multiple model systems. A, LNCaP cells were transfected with constructs encoding empty vector, T7-SRp40, or T7-SF2, harvested after 48 hours and examined for the expression of indicated transcripts. Representative PCR (left) and quantification by qPCR of at least three independent experiments (right). B, DT40-ASF/SF2 cells were transfected with plasmids encoding either empty vector or the G870 minigene. Transfected cells were split into populations treated with vehicle or doxycycline for 24 hours. Cells were harvested, lysed, and subjected to immunoblot analyses with the indicated antisera (left) or RNA was isolated and subjected to reverse transcription-PCR analyses for the indicated transcripts (right).

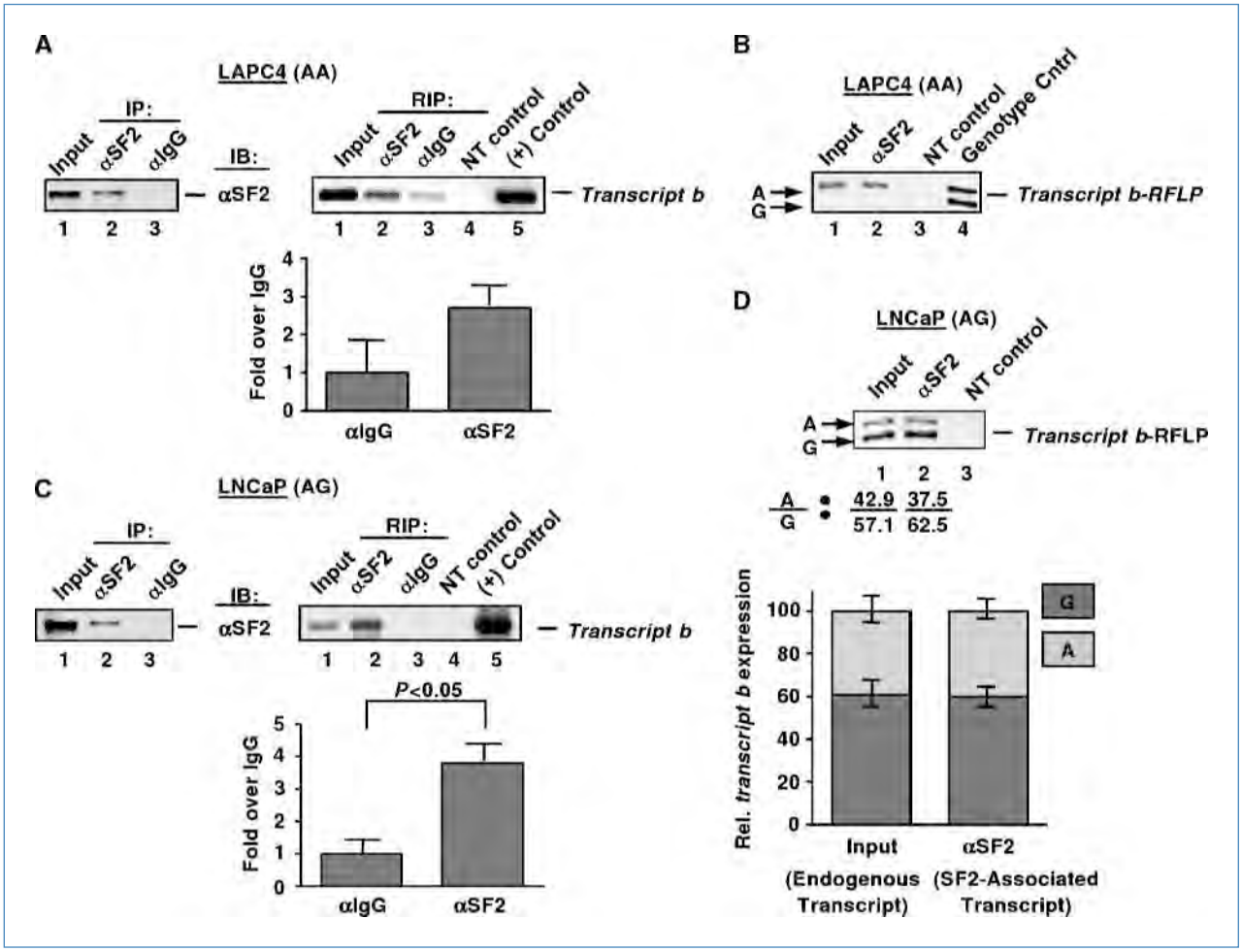

Figure 4. Endogenous SF2 can associate with transcript generated from both G/A870 alleles in PCa. A, asynchronous LAPC4 cells were harvested and subjected to a RIP. An aliquot of immunoprecipitated complexes was subjected to SDS-PAGE and immunoblot to validate SF2 recovery (left). From the remaining sample, RNA was isolated, converted to cDNA, and analyzed for the presence of *transcript-b* by PCR (representative sample, right). qPCR quantification of at least four independent experiments is shown (bottom). *P* value determined by ANOVA. B, input and SF2-recovered transcripts from A were amplified with primers required for RFLP analyses of the *CCND1* polymorphism, which have been previously described (38). Following amplification, the PCR product was ScrFI digested and visualized on an agarose gel. Genotype control is from amplification of genomic DNA from LNCaP cells. C, asynchronous LNCaP cells were treated similar to A. Relative SF2 pulldown and *transcript-b* recovery are shown. Quantification was from at least five independent RIP experiments. D, analyses of input and SF2-associated transcripts by RFLP from C is shown (top). Relative band intensities of endogenous, overall *transcript-b* levels as a function of the G/A870 allele (generated from the input band) or SF2-associated transcript (generated from the SF2-RIP studies) were quantified from at least five independent experiments (bottom). Averages and SEM are shown. The ratio of *transcript-b* as A to G (A/G) is shown for the representative image.

quantification of the SF2-bound fraction (Fig. 4D, lane 2) resulted in a similar ratio of transcript generated from the G and A alleles, based on at least five independent analyses and quantification (Fig. 4D, bottom). Thus, these data show that endogenous SF2 could associate with both transcripts when present, but shows a slight preference for the G870-derived transcript.

SF2 predominantly associates with and produces* transcript-b *from the G allele. Given the observation that elevated SF2 correlates with high cyclin D1b in human PCa and is sufficient in PCa model systems to induce cyclin D1b production, the effect of the G/A870 polymorphism for this event was determined. As shown, SF2 levels were elevated through ectopic expression of an HA-tagged allele in cells heterozygous for the polymorphism and that express low endogenous cyclin D1b (Supplementary Fig. S6), so as to mimic tumor-associated SF2 induction. Subsequent analyses (Fig. 5A) confirmed SF2-mediated enhancement of overall *transcript-b* (compare lanes 1 and 4). In addition, SF2 association with *transcript-b* was markedly enhanced in the SF2-transfected cells (compare lanes 2 and 5), demonstrating that elevated SF2 results not only in enhanced *transcript-b* production, but also enhanced SF2-associated transcript.

Furthermore, these findings also allowed determination of (*a*) the allele composition of *transcript-b* levels elevated by SF2 and (*b*) whether elevated SF2 altered the likelihood of either allele to directly associate with SF2. To answer both questions, RFLP analyses were performed with the SF2-associated

transcripts after SF2 induction or in control cells. First, it was observed that SF2 predominantly resulted in an induction of *transcript-b* from the G870 allele (Fig. 5B, lane 1 versus lane 3); quantified in C). These data implicate SF2 as an allele-selective modifier of the alternative splicing event. Second, to determine whether this action of SF2 was direct, RFLP analyses of the SF2-bound fraction was compared in cells with steady-state SF2 (control) versus those with elevated SF2 expression (Fig. 5B, compare lanes and 2 and 4). For ease of comparison, the percentage of SF2 bound to the A870-derived transcript (relative to input) in control cells was set to "1", and relative association determined for each (Table 1). As shown, SF2 association with the G allele was statistically unchanged under conditions mimicking tumor-associated SF2 elevation. By contrast, SF2 association with the A allele was enhanced by ~2-fold under these conditions.

Collectively, these data suggest a model (summarized in Fig. 6) in which SF2 elicits allele-selective effects on cyclin D1b production in prostate cancer that are dose-dependent. Under steady state conditions, SF2 binds with slight preference to transcripts generated from the G870 allele; concordantly, the G allele accounts for 61% of *transcript-b* production with the A allele accounting for the remaining 39%. These data are consistent with the supposition that SF2 acts (potentially directly) to suppress splicing at the intron 4–exon 4 boundary, and that this event is facilitated by the G870 allele. Elevated SF2, such as observed in human disease, can modestly enhance SF2 association with the A allele transcript, but induced SF2 remains predominantly associated with and produces cyclin D1b from the G allele transcript.

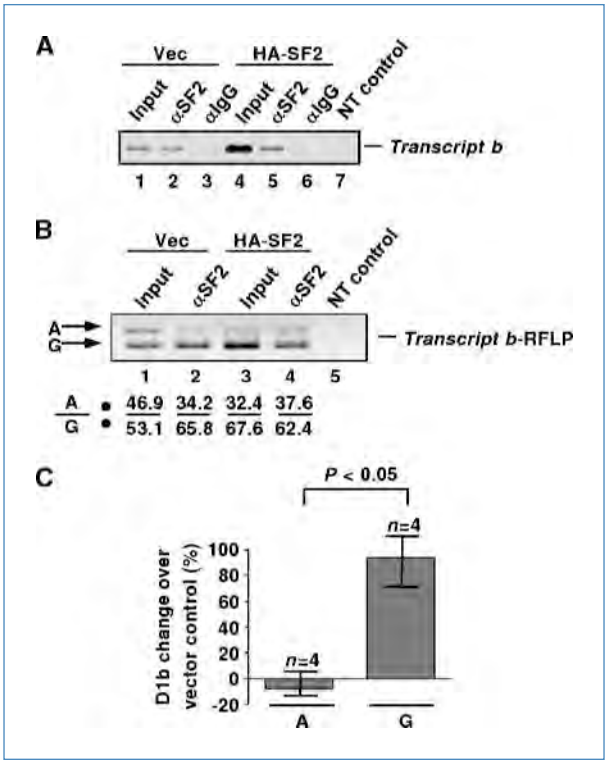

Figure 5. Elevated expression of SF2 predominately associates with and promotes *transcript-b* production from the G allele. A, LNCaP cells were transfected with expression constructs encoding empty vector or HA-SF2. Forty-eight hours later, cells were harvested and subjected to a RIP. *Transcript-b* expression was analyzed as previously described (Fig. 1). B, input and SF2-associated RNA from A were then subjected to RFLP analyses as in Fig. 4D. Representative analyses are shown with their respective ratios of *transcript-b* as A to G (A/G). C, to assess the effect of SF2 elevation on allele-specific *transcript-b* production, relative change in transcript abundance generated from the G or A allele after SF2 introduction was determined by quantification of the "input" RFLP signal (represented by B, percentage of change in lane 3 versus lane 1) from four independent experiments. Average change in relative G or A allele derived transcript per experiment was averaged and plotted as the percentage of D1b change over vector control.

Table 1. Relative SF2 association

% Allele	Control	SF2
A	1.0	1.92 ± 0.57
G	5.34 ± 0.80	4.42 ± 0.80

Discussion

Here, we identify SF2 as a dose-dependent effector of cyclin D1b production, and provide evidence of a tumor-associated mechanism that alters the influence of the G/A870 polymorphism. Although minigene analyses of individual alleles (Fig. 1) and evaluation of nonneoplastic human tissue support a role for the A870 allele in promoting *transcript-b* and resultant cyclin D1b production, the influence of the A allele is thought to be relieved in PCa (11). The present data shows that the RNA-binding protein SF2, which is induced as a function of PCa progression, predicts for cyclin D1b (not cyclin D1a) elevation in human disease (Fig. 2). By contrast, cyclin D1b production was attenuated in model systems of SF2 depletion (Fig. 3). Functional studies in PCa cells heterozygous for the polymorphism unexpectedly showed that SF2 predominantly associates with and induces *transcript-b* derived from the G870 allele; however, association with and production of *transcript-b* from the A allele could still occur (Figs. 4 and 5). Together, these studies provide a novel mechanism by which cyclin D1b oncogene production is induced in human disease, and identify tumor-associated SF2 as an allele-selective effector of cyclin D1b.

Despite the potent oncogenic activity of cyclin D1b, knowledge of the factor(s) that regulate the *CCND1* alternative splicing event are poorly defined. The present identification of SF2 as an effector of *transcript-b* and cyclin D1b production using both *in vitro* models and analyses of human tumors provides strong evidence linking splicing factor deregulation to oncogene activation. It is tempting to speculate as to whether SF2 could cooperate with few effectors of cyclin D1b production that were identified in other tissue types. For example, in colorectal cells, it was observed that knockdown of the SWI/SNF chromatin remodeling complex subunit Brahma (BRM) increased *transcript-b* production without altering *transcript-a* (34). These findings are of note,

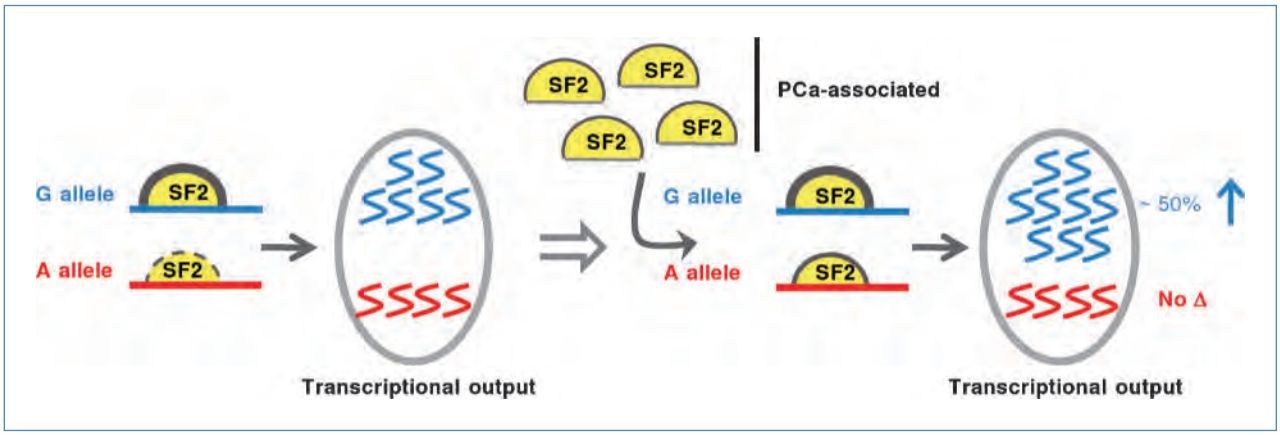

Figure 6. Model of SF2 activity. Under steady-state conditions, ~60% of detected *transcript-b* is generated from the G870 allele, and SF2 predominantly binds to the G870-derived transcript. SF2 elevation, as associated with PCa progression, enhances association with the A870 allele, but the observed increase in *transcript-b* production is exquisitely associated with the G870 allele. No Δ, no change.

as BRM was recently shown to be significantly downregulated in human PCa (35), and it has yet to be determined whether BRM loss might also affect SF2 levels. In a separate study, a chromosomal translocation-derived transcription factor known to be upregulated in Ewing's sarcoma (EWS-FLI1) was found to enhance cyclin D1b production by diminishing the rate of transcriptional elongation (10). More recently, it was shown that Sam68 promotes cyclin D1b production through a splicing-repressive mechanism by blocking U1-70k association, a constitutive spliceosome accessory factor of the U1 small nuclear ribonucleoprotein that is necessary for 5′ splice site recognition (36, 37). It is well established that chromatin remodeling complexes could alter RNA *Pol*II accessibility by alteration of the native chromatin structure (38); moreover, pre-mRNA splicing occurs cotranscriptionally, and is aided by the function of SR proteins (including SF2) which can bind to *Pol*II and selected SWI/SNF subunits (BAF155 and BAF53A; ref. 39). Therefore, an attractive hypothesis is that SF2 could act in concert with either BRM or EWS-FLI1 to modulate the *CCND1* splicing event. Given the marked protumorigenic activity of cyclin D1b, these collective observations further underscore the importance of delineating the mechanisms that regulate or influence the SF2-mediated alternative splicing event in models of disease relevance.

With regard to clinical relevance, it is notable that in PCa cells, SF2 exhibited an allele-selective effect on the alternative splicing event. Previous studies showed that in nonneoplastic tissue, the A870 allele was associated with higher *transcript-b* production but that the influence of the A allele was lost in PCa specimens (11); these findings suggested that tumor-associated factors might either bypass the effect of the polymorphism or bolster the production of *transcript-b* from the G allele. The present data supports the hypothesis that SF2 may serve as such a factor because SF2 induction (such as occurs in human disease) predominantly binds to *transcript-b* derived from the G allele and promotes the accumulation of this transcript. Production of *transcript-b* still occurs from the A allele (~40%), however, this seems to remain unaltered following modulation of SF2 expression, thus it will be of interest to examine the functional relationship between SF2 and the A allele. Examination of how SF2 influences allele-specific cyclin D1b production in other tumor types in which cyclin D1b levels are elevated as a function of tumorigenesis (e.g., colon, bladder, or breast carcinoma) will be critical (6, 9, 40, 41). Given the propensity of SF2 to preferentially bind to and induce cyclin D1b from the G allele, the present data indicate that SF2 may promote intron inclusion at the *CCND1* exon 4–intron 4 boundary, and precedence for a splicing repressor function of SF2 was previously established for the MNK2 kinase (42). It cannot be presently ruled out that the effect of SF2 could be manifested through other means, given the ability of SF2 to affect mRNA metabolism, mRNA transport and/or stability, and mTOR-mediated translation (43, 44). Future analyses will be directed at defining the action of SF2 at the exon 4–intron 4 boundary.

Finally, the present findings provide new insight into a potential means through which SF2 promotes cellular transformation. It is noteworthy that SF2 could independently induce transformation and induce tumor growth *in vivo* (42), thus demonstrating phenotypes similar to those observed with cyclin D1b (3, 5). As SF2 levels correlated with cyclin D1b in human PCa, these data implicate cyclin D1b as a possible downstream effector of SF2-mediated cellular transformation in the prostate. Additional mechanisms are predicted to contribute to this event, as perturbations in spliceosome function and RNA processing proteins have been recently identified as major contributors to genomic instability (45). Importantly, dysregulation of splicing factors has also been shown to accelerate PCa progression and metastases, such as observed by deregulated expression of SRp40, or in the presence of specific, cancer risk–associated polymorphisms in the intronic region of the KLF6 tumor suppressor that create novel SRp40 binding sites (46). Given the disease relevance of this event, it will be imperative to determine not only the consequence of SR protein dysregulation, but whether these events act in concert to promote tumor progression. Ongoing studies suggest that tumor-associated SF2 induction

may induce and enhance the migration and invasion phenotype in prostate cancer cells (data not shown), providing the impetus to discern the effect of SF2 on the development of tumor metastases.

In summary, our study identifies SF2 as a novel, clinically relevant effector of *CCND1* alternative splicing, capable of promoting allele-specific induction of cyclin D1b in prostate cancer. The findings presented are among the first to determine how the cyclin D1b oncogene is enhanced in human disease, and provide the foundation for future studies directed at developing mechanisms to target oncogene induction.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the K. Knudsen lab for ongoing discussions, Dr. E. Knudsen for critical commentary, Drs. J. Bruzik, C. Chalfant, and R. Carstens for valuable discussions, and J. Tulenko for assistance with figure presentation.

Grant Support

NIH grants R01 CA09996 and CA116777 (K.E. Knudsen), Associazione Italiana Ricerca sul Cancro and Association for International Cancer Research (C. Sette), and a DOD New Investigator Award (C.E.S. Comstock). M.J. Schiewer and M.A. Augello were supported by predoctoral fellowships from the Department of Defense (PC094195 to M.J. Schiewer and PC094596 to M.A. Augello), and N.A. Olshavsky is supported by NIEHS training grant 2T32ES007250-21A1.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/21/2009; revised 03/10/2010; accepted 03/10/2010; published OnlineFirst 05/11/2010.

References

1. Betticher DC, Thatcher N, Altermatt HJ, Hoban P, Ryder WD, Heighway J. Alternate splicing produces a novel cyclin D1 transcript. Oncogene 1995;11:1005–11.
2. Hosokawa Y, Tu T, Tahara H, Smith AP, Arnold A. Absence of cyclin D1/PRAD1 point mutations in human breast cancers and parathyroid adenomas and identification of a new cyclin D1 gene polymorphism. Cancer Lett 1995;93:165–70.
3. Rojas P, Benavides F, Blando J, et al. Enhanced skin carcinogenesis and lack of thymus hyperplasia in transgenic mice expressing human cyclin D1b (CCND1b). Mol Carcinog 2009;48:508–16.
4. Solomon DA, Wang Y, Fox SR, et al. Cyclin D1 splice variants. Differential effects on localization, RB phosphorylation, and cellular transformation. J Biol Chem 2003;278:30339–47.
5. Lu F, Gladden AB, Diehl JA. An alternatively spliced cyclin D1 isoform, cyclin D1b, is a nuclear oncogene. Cancer Res 2003;63:7056–61.
6. Kim CJ, Nishi K, Isono T, et al. Cyclin D1b variant promotes cell invasiveness independent of binding to CDK4 in human bladder cancer cells. Mol Carcinog 2009;10:953–64.
7. Burd CJ, Petre CE, Morey LM, et al. Cyclin D1b variant influences prostate cancer growth through aberrant androgen receptor regulation. Proc Natl Acad Sci U S A 2006;103:2190–5.
8. Li R, An SJ, Chen ZH, et al. Expression of cyclin D1 splice variants is differentially associated with outcome in non-small cell lung cancer patients. Hum Pathol 2008;39:1792–801.
9. Millar EK, Dean JL, McNeil CM, et al. Cyclin D1b protein expression in breast cancer is independent of cyclin D1a and associated with poor disease outcome. Oncogene 2009;28:1812–20.
10. Sanchez G, Bittencourt D, Laud K, et al. Alteration of cyclin D1 transcript elongation by a mutated transcription factor up-regulates the oncogenic D1b splice isoform in cancer. Proc Natl Acad Sci U S A 2008;105:6004–9.
11. Comstock CE, Augello MA, Benito RP, et al. Cyclin D1 splice variants: polymorphism, risk, and isoform-specific regulation in prostate cancer. Clin Cancer Res 2009;17:5338–49.
12. Holley SL, Heighway J, Hoban PR. Induced expression of human CCND1 alternative transcripts in mouse Cyl-1 knockout fibroblasts highlights functional differences. Int J Cancer 2005;114:364–70.
13. Howe D, Lynas C. The cyclin D1 alternative transcripts [a] and [b] are expressed in normal and malignant lymphocytes and their relative levels are influenced by the polymorphism at codon 241. Haematologica 2001;86:563–9.
14. Petre CE, Wetherill YB, Danielsen M, Knudsen KE. Cyclin D1: mechanism and consequence of androgen receptor co-repressor activity. J Biol Chem 2002;277:2207–15.
15. Petre-Draviam CE, Cook SL, Burd CJ, Marshall TW, Wetherill YB, Knudsen KE. Specificity of cyclin D1 for androgen receptor regulation. Cancer Res 2003;63:4903–13.
16. Petre-Draviam CE, Williams EB, Burd CJ, et al. A central domain of cyclin D1 mediates nuclear receptor corepressor activity. Oncogene 2005;24:431–44.
17. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 1987;7:2745–52.
18. Wang J, Takagaki Y, Manley JL. Targeted disruption of an essential vertebrate gene: ASF/SF2 is required for cell viability. Genes Dev 1996;10:2588–99.
19. Caceres JF, Misteli T, Screaton GR, Spector DL, Krainer AR. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J Cell Biol 1997;138:225–38.
20. Lai MC, Tarn WY. Hypophosphorylated ASF/SF2 binds TAP and is present in messenger ribonucleoproteins. J Biol Chem 2004;279:31745–9.
21. Burd CJ, Petre CE, Moghadam H, Wilson EM, Knudsen KE. Cyclin D1 binding to the androgen receptor (AR) NH2-terminal domain inhibits activation function 2 association and reveals dual roles for AR corepression. Mol Endocrinol 2005;19:607–20.
22. Sawa H, Ohshima TA, Ukita H, et al. Alternatively spliced forms of cyclin D1 modulate entry into the cell cycle in an inverse manner. Oncogene 1998;16:1701–12.
23. Paronetto MP, Achsel T, Massiello A, Chalfant CE, Sette C. The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. J Cell Biol 2007;176:929–39.
24. Wang Y, Dean JL, Millar EK, et al. Cyclin D1b is aberrantly regulated in response to therapeutic challenge and promotes resistance to estrogen antagonists. Cancer Res 2008;68:5628–38.
25. Jensen FC, Girardi AJ, Gilden RV, Koprowski H. Infection of human and simian tissue cultures with rous sarcoma virus. Proc Natl Acad Sci U S A 1964;52:53–9.
26. Webber MM, Bello D, Kleinman HK, Hoffman MP. Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. Carcinogenesis 1997;18:1225–31.
27. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: a web resource to identify exonic splicing enhancers. Nucleic Acids Res 2003;31:3568–71.
28. Smith PJ, Zhang C, Wang J, Chew SL, Zhang MQ, Krainer AR. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Hum Mol Genet 2006;15:2490–508.
29. Lapointe J, Li C, Higgins JP, et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A 2004;101:811–6.
30. Vanaja DK, Cheville JC, Iturria SJ, Young CY. Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. Cancer Res 2003;63:3877–82.
31. Welsh JB, Sapinoso LM, Su AI, et al. Analysis of gene expression

identifies candidate markers and pharmacological targets in prostate cancer. Cancer Res 2001;61:5974–8.
32. Crowley CW, Cohen RL, Lucas BK, Liu G, Shuman MA, Levinson AD. Prevention of metastasis by inhibition of the urokinase receptor. Proc Natl Acad Sci U S A 1993;90:5021–5.
33. Li X, Wang J, Manley JL. Loss of splicing factor ASF/SF2 induces G2 cell cycle arrest and apoptosis, but inhibits internucleosomal DNA fragmentation. Genes Dev 2005;19:2705–14.
34. Batsche E, Yaniv M, Muchardt C. The human SWI/SNF subunit Brm is a regulator of alternative splicing. Nat Struct Mol Biol 2006;13:22–9.
35. Shen H, Powers N, Saini N, et al. The SWI/SNF ATPase Brm is a gatekeeper of proliferative control in prostate cancer. Cancer Res 2008;68:10154–62.
36. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem 2003;72:291–336.
37. Paronetto MP, Cappellari M, Busa R, et al. Alternative splicing of the cyclin D1 proto-oncogene is regulated by the RNA-binding protein Sam68. Cancer Res 70:229–39.
38. Clapier CR, Cairns BR. The biology of chromatin remodeling complexes. Annu Rev Biochem 2009;78:273–304.
39. Das R, Yu J, Zhang Z, et al. SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. Mol Cell 2007;26:867–81.
40. Bala S, Peltomaki P. CYCLIN D1 as a genetic modifier in hereditary nonpolyposis colorectal cancer. Cancer Res 2001;61:6042–5.
41. Hong Y, Eu KW, Seow-Choen F, Fook-Chong S, Cheah PY. GG genotype of cyclin D1 G870A polymorphism is associated with increased risk and advanced colorectal cancer in patients in Singapore. Eur J Cancer 2005;41:1037–44.
42. Karni R, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. Nat Struct Mol Biol 2007;14:185–93.
43. Michlewski G, Sanford JR, Caceres JF. The splicing factor SF2/ASF regulates translation initiation by enhancing phosphorylation of 4E-BP1. Mol Cell 2008;30:179–89.
44. Zhong XY, Wang P, Han J, Rosenfeld MG, Fu XD. SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. Mol Cell 2009;35:1–10.
45. Paulsen RD, Soni DV, Wollman R, et al. A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. Mol Cell 2009;35:228–39.
46. Narla G, Difeo A, Reeves HL, et al. A germline DNA polymorphism enhances alternative splicing of the KLF6 tumor suppressor gene and is associated with increased prostate cancer risk. Cancer Res 2005;65:1213–22.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 10, pp. 8117–8127, March 11, 2011
Printed in the U.S.A.

Cyclin D1 Is a Selective Modifier of Androgen-dependent Signaling and Androgen Receptor Function*[S]

Received for publication, August 3, 2010, and in revised form, December 27, 2010 Published, JBC Papers in Press, January 5, 2011, DOI 10.1074/jbc.M110.170720

Clay E. S. Comstock[‡§], Michael A. Augello[‡§], Matthew J. Schiewer[‡§], Jason Karch[¶], Craig J. Burd[‖], Adam Ertel[‡§], Erik S. Knudsen[‡§], Walter J. Jessen[**], Bruce J. Aronow[‡‡], and Karen E. Knudsen[‡§ §§¶¶1]

From the [‡]Kimmel Cancer Center, [§]Department of Cancer Biology, [§§]Department of Urology, and [¶¶]Department of Radiation Oncology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, the [¶]Department of Cell and Cancer Biology, University of Cincinnati, Cincinnati, Ohio 45221, [‖]NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709, the [**]Covance Biomarker Center of Excellence, Greenfield, Indiana 46140, and [‡‡]Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229

D-type cyclins regulate cellular outcomes in part through cyclin-dependent, kinase-independent mechanisms that modify transcription factor action, and recent *in vivo* studies showed that cyclin D1 associates with a large number of transcriptional regulators in cells of the retina and breast. Given the frequency of cyclin D1 alterations in cancer, it is imperative to delineate the molecular mechanisms by which cyclin D1 controls key transcription factor networks in human disease. Prostate cancer was used as a paradigm because this tumor type is reliant at all stages of the disease on androgen receptor (AR) signaling, and cyclin D1 has been shown to negatively modulate AR-dependent expression of prostate-specific antigen (*KLK3/PSA*). Strategies were employed to control cyclin D1 expression under conditions of hormone depletion, and the effect of cyclin D1 on subsequent androgen-dependent gene expression was determined using unbiased gene expression profiling. Modulating cyclin D1 conferred widespread effects on androgen signaling and revealed cyclin D1 to be a selective effector of hormone action. A subset of androgen-induced target genes, known to be directly regulated by AR, was strongly suppressed by cyclin D1. Analyses of AR occupancy at target gene regulatory loci of clinical relevance demonstrated that cyclin D1 limits AR residence after hormone stimulation. Together, these findings reveal a new function for cyclin D1 in controlling hormone-dependent transcriptional outcomes and demonstrate a pervasive role for cyclin D1 in regulating transcription factor dynamics.

The D-type cyclins (cyclins D1, D2, and D3) utilize pleiotropic functions to elicit cellular outcomes and are frequently altered in the course of human cancer (1–4). A well characterized function of D-cyclins in many model systems is their ability to associate with and activate cyclin-dependent kinase 4 or 6 (CDK4 or -6)[2] to initiate proliferative phenotypes (5–7). Evidence has revealed that reconstituting individual D-cyclins in fibroblasts lacking cyclins D1, D2, and D3 may result in distinct functions (8). Interestingly, in this system, cyclin D1 failed to confer significant CDK4 kinase activity, suggesting that cyclin D1 may have functions in addition to cell cycle control (9). These findings are consistent with robust *in vitro* and *in vivo* findings that revealed the existence of "kinase-independent" cyclin D1 activities (1).

The kinase-independent functions of cyclin D1 have significant consequence for both tissue development and tumor biology (2, 4, 10, 11). First, it is notable that D-type cyclins and associated CDKs are dispensable for cellular proliferation (12, 13). Second, retinal and mammary hypoplasia observed in cyclin $D1^{-/-}$ mice can be rescued by knock-in of a mutant allele, defective in the ability to activate CDK4, indicating that selected developmental requirements for cyclin D1 may be kinase-independent (14). Third, recent unbiased, *in vivo* analysis of cyclin D1 complexes showed that endogenous cyclin D1 is found in complex with a large number of sequence specific transcription factors (15). In fact, transcriptional regulators represented the most prevalent class of protein found in association with cyclin D1. Subsequent ChIP-chip analyses showed that in the retina, cyclin D1 is found associated with chromatin and that disruption of cyclin D1 function results in critical, tissue-specific effects on gene transcription. These findings have drawn significant interest and support previous studies demonstrating that perturbation of cyclin D1-mediated transcriptional control impacts human cancers. For example, the ability of cyclin D1 to bind and regulate C/EBPβ impacts clinical outcomes in breast cancer (16). In the context of PCa, cyclin D1 has been shown to influence the response to anoikis through association with FOXO1 (17). Cell cycle progression can also be altered through kinase-independent mechanisms because cyclin D1 antagonizes the antiproliferative effects of DMP1 through direct association (18). Last, cyclin D1 has been shown to interact with and modulate several nuclear receptors of critical importance for hormone-dependent cancers, including estrogen receptor (19, 20), thyroid hormone receptor (21), per-

* This work was supported, in whole or in part, by National Institutes of Health Grant CA099996 (to K. E. K.). This work was also supported by Department of Defense New Investigator Award PC094507 (to C. E. S. C.) and Department of Defense Predoctoral Fellowships PC094195 (to M. J. S.) and PC094596 (to M. A. A.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.

[1] To whom correspondence should be addressed: 233 S. 10th St., BLSB 1008A, Philadelphia, PA 19107. Tel.: 215-503-8574; Fax: 215-923-4498; E-mail: Karen.Knudsen@kimmelcancercenter.org.

[2] The abbreviations used are: CDK, cyclin-dependent kinase; PCa, prostate cancer; AR, androgen receptor; PSA, prostate-specific antigen; AROR, AR-occupied region; ARE, androgen response element; DHT, dihydrotestosterone; TSS, transcription start site; Ad, adenovirus; qPCR, quantitative PCR.

oxisome proliferator-activated receptor γ (22), and the androgen receptor (AR) (23, 24). Taken together, these observations indicate that cyclin D1 plays an important role in regulating transcriptional factor activity.

Previous investigation revealed that cross-talk between AR and cyclin D1 serves as a rheostat to modulate mitogen-mediated AR signaling (22) and that this process may be disrupted in PCa (25–27). Ligand-activated AR initiates signaling events that result in the mTOR-dependent induction of cyclin D1 translation (26, 28). Accumulated cyclin D1 protein acts both to initiate CDK4 activation (promoting G_1-S transition) and to dampen further AR activation through direct and CDK-independent association with the receptor. Through these means, cyclin D1 appears to serve as a mechanism to control the strength and duration of mitogenic signaling in the presence of androgen. The ability of cyclin D1 to govern AR transcriptional activity has been extensively studied using the well known AR target gene *KLK3/PSA* (29). Molecular analyses demonstrated that cyclin D1 engages at least two mechanisms to suppress ligand-dependent AR activity. First, cyclin D1 binds to the F*XX*LF motif of AR to block ligand-induced conformational changes in the receptor (N-C-terminal interaction) that foster transactivation potential (30). Second, cyclin D1 is known to associate with a select group of histone deacetylases (21, 31–33), and this activity is essential for robust suppression of ligand-stimulated AR activity (34).

The importance of cyclin D1-mediated AR regulation is underscored by recent studies addressing both the cellular and clinical relevance. Investigation of human prostatic adenocarcinomas showed a large percentage of specimens with low or undetectable cyclin D1 expression, and tumors lacking cyclin D1 have been shown to be associated with elevated serum PSA (27), suggestive of increased AR activity. Strikingly, a significant subset of tumors examined had elevated cyclin D1b (a variant of cyclin D1) (26), which has compromised AR-regulatory capacity (25). Thus, the ability of cyclin D1 to suppress AR activity appears to be diminished in PCa, consistent with the role of AR in promoting tumor development and progression (35). Conversely, introduction of the isolated cyclin D1 domain (repressor domain) responsible for transcriptional regulation of AR revealed that this functional motif is sufficient to attenuate ligand-dependent AR activity, cooperate with AR-directed therapeutics, and reduce cell viability in AR-dependent PCa cells (36). Combined, these findings identify cyclin D1 as a major effector of AR function and cellular outcomes in PCa.

Given the importance of cyclin D1 as a transcriptional regulator of AR in PCa, an unbiased approach was utilized to assess the overall impact of cyclin D1 on androgen-responsive gene expression and AR function at endogenous target gene sites. These studies unexpectedly revealed that cyclin D1 serves as a selective modifier of androgen activity, capable of both suppressing and facilitating androgen-dependent gene expression. However, genes that are known to be directly regulated by AR were suppressed by cyclin D1, indicating that this is a primary means of AR modulation. Subsequent analyses identified that cyclin D1 limits ligand-induced AR residence on chromatin, thus illuminating additional mechanisms of cyclin D1 action. Together, these findings provide critical insight into the means by which cyclin D1 controls AR function and the response to androgen stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The androgen-dependent prostate cancer cell lines (LNCaP and VCaP) were maintained as previously described (37). To examine transcriptional outcome, LNCaP or VCaP ($2.9 \times 10^4/cm^2$) cells were plated on poly-L-lysine in 5% charcoal-dextran-treated serum (HyClone) for 72 h. Cells were transduced (12 h) with either Ad-GFP control or Ad-cyclin D1 and subsequently treated (18 h) with ethanol (0.1%) or a physiological dose of dihydrotestosterone (DHT) (1 nM) (38). Cyclin D1-transduced cells treated with ethanol were included in the validation experiments to assess the impact of cyclin D1 on basal transcription. RNA was isolated using the standard TRIzol method and was either subjected directly to microarray analysis or converted to cDNA for gene expression analysis. RNA interference (RNAi) was performed using LNCaP ($8.6 \times 10^4/cm^2$) cells plated on poly-L-lysine and maintained (24 h) in standard growth conditions. Then cells were transfected overnight (16 h) in serum-free conditions with a control or *CCND1* siRNA (D-001810-10-20 or L-003210-00-0020, respectively; Thermo Scientific) according to the manufacturer's specifications and then incubated with standard growth conditions and harvested for analysis at the indicated times.

Microarray Analysis and Bioinformatics—Microarray analysis was performed as follows. Total RNA samples (0.5 μg) for each treatment condition ($n = 3$), as described above, were labeled using the standard labeling protocol (small scale protocol version 2.0) and hybridized to HG-U133plus2 GeneChips (Affymetrix). GeneChips were quantified with an Affymetrix Gene Array Scanner (software version 1.4, default settings), and then "CEL" files were generated using Affymetrix Microarray Suite 5.0. Individual samples were normalized using the robust multichip analysis algorithm as implemented in Bioconductor/R. Normalized data were refined using a custom chip definition file based on target definitions (Hs133 REFSEQ version 8, represented by 26,183 transcripts) to provide a more accurate interpretation of the expression data (39). The data set (.CEL files) is available in the online Gene Expression Omnibus (GEO) repository (accession number GSE26483). All statistical comparisons and visualizations were performed using GeneSpring GX version 7.3.1 (Agilent). Androgen-regulated transcripts were identified using a *t* test ($p \leq 0.05$) between control-transduced LNCaP cells treated with ethanol or DHT. Androgen-regulated transcripts were filtered using a 1.2-fold cut-off and then overlaid with the corresponding expression values in the presence of cyclin D1 and DHT. To identify expression patterns, the transcripts were empirically assigned to clusters using the *k*-means clustering algorithm. Statistically overrepresented functional annotations were identified using the GOterm biological processes setting in the Web-based Database for Annotation, Visualization, and Integrated Discovery (DAVID). Assessment of the presence or absence of androgen receptor-occupied regions (ARORs) within 50 kb of transcriptional start sites (TSSs) was performed by uploading a published (40) and publicly available ChIP-seq data set from

LNCaP cells into the University of California Santa Cruz Genome Browser on the NCBI36/Hg18 (March 2006) assembly (41). The TSS for individual transcripts from the microarray expression data set was determined by submitting the transcript accession numbers in batch mode to MatchMiner (42).

Gene Expression Analysis—Independent validation of the microarray expression profile was performed with cDNA generated from RNA (5 μg) using the Superscript system (Invitrogen). Conventional PCR analysis and oligonucleotides for *KLK3/PSA* and *GAPDH* have been described previously (43). Briefly, conventional PCR for *KLK3/PSA* and *GAPDH* was performed at 26 cycles. Products were resolved on agarose (2%) and visualized with ethidium bromide. The quantitative PCR method and Taqman assays for *KLK3/PSA* have been described previously (26), whereas the relative expression of all other transcripts normalized to *GAPDH* (oligonucleotides are described in supplemental Table 2 except for the *TMPRSS2* primers that have been previously described (44)) was performed using Power SYBR Green and a StepOne Machine (Applied Biosystems). Validation of transcripts is represented as the mean -fold change ± S.E. of 3–4 individual experiments where each condition within an experiment is the average of two technical replicates. Statistics were determined by analysis of variance, and significance ($p \leq 0.05$) was calculated using Tukey's multiple comparison test using GraphPad Prism version 4.

Immunoblot Analysis—Representative LNCaP cell lysates (40 μg), treated as described above, were separated by polyacrylamide gel electrophoresis to evaluate cyclin D1 protein expression. Gels were transferred to PVDF and immunoblotted (1:1000) for cyclin D1 (NeoMarkers, catalog no. AB-3), GFP (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), catalog no. SC-9996), and loading control β-Tubulin (Santa Cruz Biotechnology, Inc., catalog no. SC-5274).

ChIP Analysis—ChIP assays for AR occupancy were performed according to a method described previously (40). LNCaP cells were treated as described above, except cells were stimulated with 10 nM DHT for 1–3 h. Genomic DNA was used for conventional PCR, as described above, with oligonucleotides for the enhancer regions of *KLK3/PSA* (ARE III) and *TMPRSS2* (ARE V) as described previously (45). Quantitative PCR was performed, as described above, except using ExpressSYBR® Green-ER/ROX mix (Invitrogen). Relative occupancy was calculated according to the following: $\Delta Ct = Ct$ (of immunoprecipitation or input) $- Ct$ (of IgG); $\Delta\Delta Ct = \Delta Ct$ (of treated) $- \Delta Ct$ (of control); occupancy $= 2^{-\Delta\Delta Ct}$.

RESULTS

Cyclin D1 Expression and Function Can Be Reconstituted after Hormone Depletion—To discern the role of cyclin D1 in controlling the transcriptional response to androgen, model systems were developed to rigorously control cyclin D1 expression (26). Androgen-dependent, AR-positive prostate cancer cells (LNCaP) were utilized because these cells lack cyclin D2 (46–49) and arrest tightly in G_0/G_1 after hormone depletion with accompanying loss of cyclin D1 and cyclin D3 expression (28, 37, 50). The impact of hormone depletion on D-cyclin expression was recapitulated herein (supplemental Fig. 1, *A* and *B*, *lanes 1* and *2*). Suppression of endogenous D-cyclins is critical because loss of a single D-type cyclin can result in partial compensation by remaining family members (51). Following hormone deprivation, cells were transduced with adenovirus encoding either GFP control (Fig. 1*A*, *lanes 1* and *2*) or cyclin D1 (*lanes 3* and *4*) and then stimulated with vehicle (ethanol) control or physiologic levels of androgen (DHT) (38). Stimulation with androgen after reconstitution restored cyclin D1 levels in the absence of androgen and prior to DHT-mediated accumulation of endogenous cyclin D1 expression, thereby allowing assessment of cyclin D1 function in the absence of the other D-type cyclins. The impact of cyclin D1 reconstitution was determined by monitoring mRNA levels of the AR target gene *KLK3/PSA* because the role of cyclin D1 in suppressing androgen-induced *KLK3/PSA* expression has been well established (29). As expected, DHT stimulation resulted in marked induction of *KLK3/PSA* expression (Fig. 1*B*, *left*, compare *lanes 1* and *2*). Notably, DHT-mediated induction of *KLK3/PSA* expression was attenuated upon cyclin D1 reconstitution (45.2%, $p < 0.05$), as determined by quantitative PCR (Fig. 1*B*, *right*). In contrast, cyclin D1 had minimal impact on basal *KLK3/PSA* expression, reinforcing the postulate that cyclin D1 can alter the transcriptional response to androgen. Similar results were observed using transfected, rather than transduced, cyclin D1 (supplemental Fig. 1*B*), similar to previous reports (25, 52). The suppressive effect of cyclin D1 on *KLK3/PSA* expression was not limited to a single cell type because

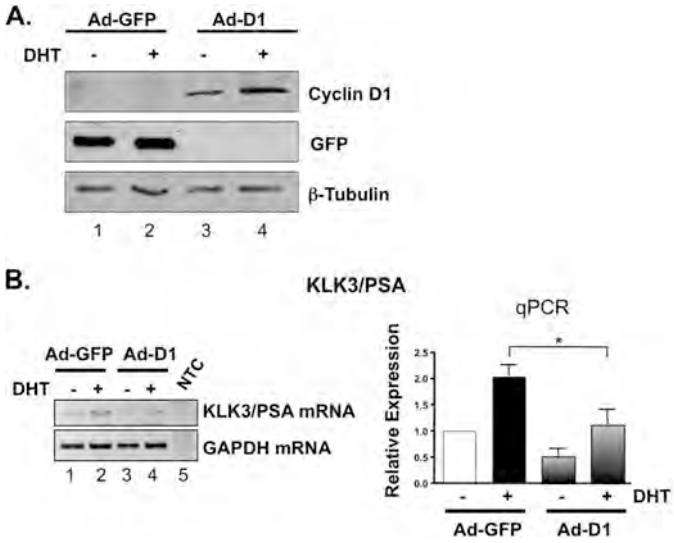

FIGURE 1. **Cyclin D1 regulates prostate-specific tumor marker expression.** To examine the cyclin D1-regulated transcriptional outcome in response to androgen, LNCaP prostate cancer cells were incubated in 5% charcoal-dextran-treated serum to naturally deplete D-type cyclins and then transduced with cyclin D1 (Ad-D1) or control (Ad-GFP) and subsequently treated for 18 h with a physiological dose of androgen (DHT; 1 nM) or ethanol (EtOH) control. *A*, to evaluate cyclin D1 protein expression, representative LNCaP cell lysates treated as described above were immunoblotted for cyclin D1, GFP, and loading control β-tubulin. Note that the post-transcriptional induction of cyclin D1 protein by androgen is maintained under cyclin D1-reconstituted conditions (*lanes 3* and *4*). *B*, *KLK3/PSA* expression was determined by conventional PCR (*left*) and Taqman-based qPCR (*right*) to assess the ability of cyclin D1 to regulate prostate-specific tumor marker expression. *NTC*, non-template control. The bar graph shows the mean-fold change ± S.E. (*error bars*) of three independent experiments, where each control sample (Ad-GFP + EtOH) is set to 1. Significant ($p < 0.05$) down-regulation by cyclin D1 of androgen-induced expression is indicated by an *asterisk*.

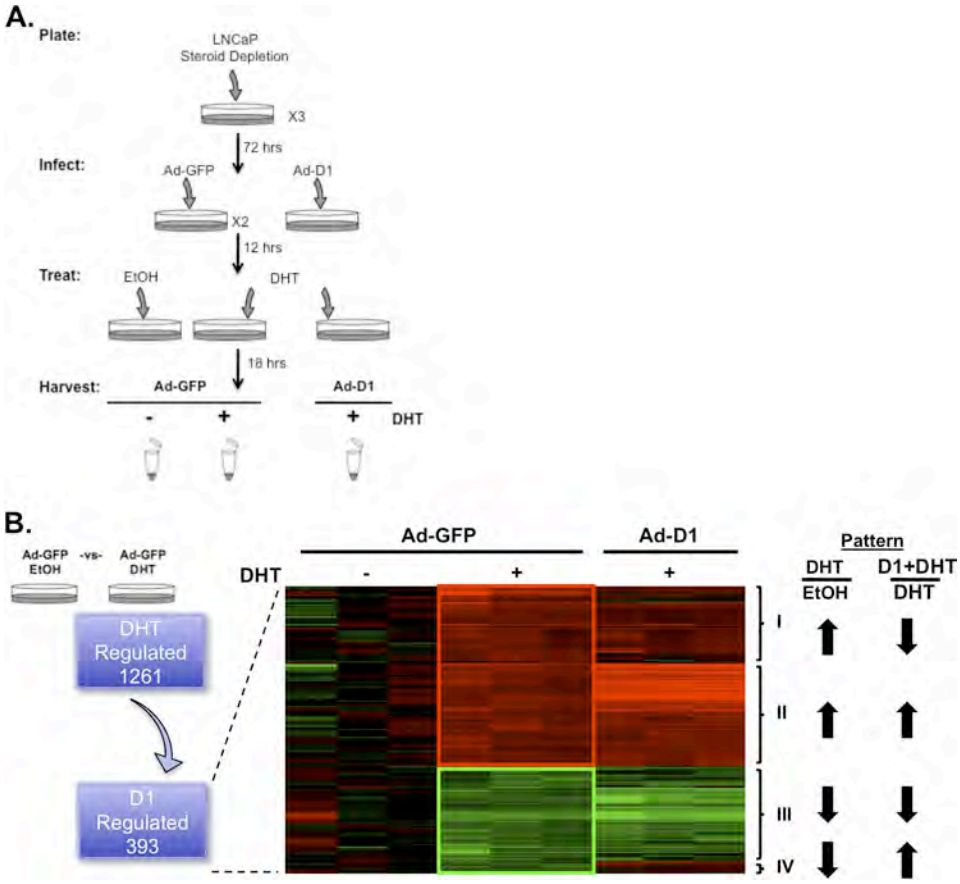

FIGURE 2. **Cyclin D1 modulates androgen-dependent gene expression.** *A*, experimental design for unbiased gene expression array analyses to identify androgen-regulated transcripts that are sensitive to cyclin D1. Treatment conditions are indicated and fully described under "Experimental Procedures"and "Results." Microarray analysis was performed in triplicate for each treatment condition on the HG-U133plus2 platform (Affymetrix). Individual samples were normalized and evaluated using a custom GeneChip library file to provide a more accurate interpretation of the expression data. All statistical comparisons and visualizations were performed using GeneSpring GX version 7.3.1 (Agilent). *B*, *schematic*, to identify androgen-regulated transcripts responsive to cyclin D1, a statistical ($p < 0.05$) comparison between GFP-transduced LNCaP cells treated with EtOH or DHT was performed. Transcripts were then selected using a 1.2-fold cut-off, and the corresponding expression values in the presence of cyclin D1 and DHT are shown. *Heat map*, to identify expression patterns (as indicated, *Patterns I–IV*), transcripts were empirically assigned to clusters using a *k*-means algorithm. *Red* and *green* indicate up-regulated and down-regulated transcripts, respectively. The androgen response and influence of cyclin D1 on the androgen response are indicated by *arrows*.

similar results were observed in a second androgen-dependent, AR-positive PCa model system, VCaP (supplemental Fig. 2). Thus, rapid cyclin D1 reconstitution (either by transduction or transfection) under hormone-depleted conditions can be used as a means to assess the impact of individual D-type cyclins on transcriptional outcomes.

Cyclin D1 Differentially Regulates Androgen-sensitive Gene Expression—Because the data above demonstrated that cyclin D1 activity can be effectively reconstituted after hormone deprivation, this model system afforded the opportunity to discern the overall impact of cyclin D1 on androgen-responsive gene expression in an unbiased manner. Such analyses are crucial because the current understanding of cyclin D1-mediated control of AR function has been largely limited to assessment of *KLK3/PSA* regulation. To determine the overall impact of cyclin D1, cells were cultured for 72 h in the absence of androgen to deplete D-cyclins and then, following cyclin D1 reconstitution, were stimulated with 1 nM DHT for 18 h (as depicted in Fig. 2*A*). Validation of cyclin D1 mRNA levels pre- and post-transduction was conducted (supplemental Fig. 3*A*), and gene expression analysis was performed using biological replicates on the Affymetrix Human Genome U133plus2 platform. Following confirmation of cyclin D1 RNA levels on the microarray (supplemental Fig. 3*B*), a custom GeneChip library file based on REFSEQ target definitions was used to provide accurate interpretation of GeneChip data (39). Initially, 1,261 transcripts were identified that were significantly ($p < 0.05$) altered by androgen stimulation compared with control, GFP-transduced cells (Fig. 2*B*, *left*). However, upon cyclin D1 reconstitution, only a subset of androgen-responsive transcripts ($n = 393$ transcripts, 257 up-regulated and 136 down-regulated) proved sensitive to cyclin D1 status. The complete list of androgen-sensitive, cyclin D1-regulated transcripts is provided in supplemental Fig. 4. Combined, these findings provided the first indication that cyclin D1 regulates a distinct subset of androgen-responsive genes in the context of PCa.

Closer examination of the 393 androgen-regulated, cyclin D1-sensitive transcripts was facilitated by *k*-means clustering analyses. As depicted in the heat map (Fig. 2*B*, *right*), four distinct cyclin D1-responsive patterns were identified. Unexpectedly, these studies revealed that cyclin D1 could antagonize (Patterns I and IV, $n = 127$ and 54, respectively) or act in concert (Patterns II and III, $n = 130$ and 82, respectively) with androgen to regulate gene networks. Gene ontology analyses of

TABLE 1
Top transcripts regulated by DHT and cyclin 1

	Symbol	Gene Name	Accession ID	Δ with DHT[a]	p-value	Δ with D1[b]
	KLK2	Kallikrein 2, prostatic, variants: 1&2	NM:005551;001002231	2.58	0.0122	1.36
		Kallikrein 2, prostatic, variant: 3	NM001002232	2.56	0.0121	1.36
	KLK3	Kallikrein 3, variants: 1&4 (prostate specific antigen)	NM:001648;001030048	2.27	0.0250	1.11
		Kallikrein 3, variant: 3 (prostate specific antigen)	NM001030047	2.25	0.0240	1.11
		Kallikrein 3, variant: 5 (prostate specific antigen)	NM001030049	2.25	0.0249	1.09
	TRPM8	Transient receptor potential cation channel, subfamily M 8	NM024080	2.16	0.0178	0.93
	ABCC4	ATP-binding cassette, sub-family C, member 4	NM005845	2.08	0.0143	1.34
	ST6GALNAC1	ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 1	NM018414	2.06	0.0368	1.13
Pattern I	ELOVL7	ELOVL family member 7, elongation of long chain fatty acids	NM024930	1.97	0.0077	1.47
	LOC642083	Hypothetical protein	XM942728	1.81	0.0218	1.56
	KLK4	Kallikrein 4 (prostase, enamel matrix, prostate)	NM004917	1.77	0.0402	0.94
	GNMT	Glycine N-methyltransferase	NM018960	1.74	0.0397	1.49
	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	NM000693	1.55	0.0404	1.22
	HOMER2	Homer homolog 2, variants: 1-4	NM:004839;199330;199331;199332	1.54	0.0062	1.23
	RAB3B	RAS oncogene family	NM002867	1.54	0.0149	1.15
	DTL	Denticleless homolog	NM016448	2.16	0.0110	4.93
	CDC6	Cell division cycle 6 homolog	NM001254	2.14	0.0339	5.10
	MCM10	Minichromosome maintenance deficient 10, variants: 1&2	NM:182751;018518	1.88	0.0330	4.75
	DEPDC1	DEP domain containing 1	NM017779	1.74	0.0165	5.08
	E2F8	E2F transcription factor 8	NM024680	1.69	0.0356	5.30
	CDCA7	Cell division cycle associated 7, variants: 1&2	NM:031942;145810	1.63	0.0156	2.90
Pattern II	MCM4	Minichromosome maintenance deficient 4, variants: 1&2	NM:005914;182746	1.62	0.0081	3.46
	PRIM2A	Primase, polypeptide 2A, 58kDa	NM000947	1.61	0.0275	2.26
	ATAD2	ATPase family, AAA domain containing 2	NM014109	1.61	0.0453	3.75
	C16orf75	Chromosome 16 open reading frame 75	NM152308	1.57	0.0054	2.72
	C11orf82	Chromosome 11 open reading frame 82	NM145018	1.56	0.0300	3.98
	BRIP1	BRCA1 interacting protein C-terminal helicase 1	NM032043	1.52	0.0010	2.41
	SI	Sucrase-isomaltase	NM001041	-1.72	0.0034	-2.30
	MYLK	Myosin, light polypeptide kinase, variants: 1,2,3A&B,4-8	NM:053025;053026;053027 NM:053028;053029;053030 NM:005965;053031;053032	-1.70	0.0399	-2.20
	MYRIP	Myosin VIIA and Rab interacting protein	NM015460	-1.52	0.0334	-1.97
	COL5A2	Collagen, type V, α2	NM000393	-1.49	0.0205	-1.96
	ST6GALNAC5	ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 5	NM030965	-1.44	0.0385	-1.77
Pattern III	HACL1	2-hydroxyacyl-CoA lyase 1	NM012260	-1.43	0.0023	-1.85
	LOC338758	Hypothetical protein	XM:931359;944677	-1.40	0.0211	-1.61
	MBD5	Methyl-CpG binding domain protein 5	NM018328	-1.33	0.0178	-1.77
	HECTD2	HECT domain containing 2	NM182765	-1.31	0.0138	-1.67
	SELENBP1	Selenium binding protein 1	NM003944	-1.28	0.0066	-1.71
	TP53INP2	Tumor protein p53 inducible nuclear protein 2	NM021202	-1.28	0.0230	-1.65
	STXBP1	Syntaxin binding protein 1, variants: 1&2	NM:003165;001032221	-1.27	0.0248	-1.73
	LOC440731	Hypothetical protein: variants: 2&4	XM:933693;944728	-1.57	0.0229	-1.31
	DDEF2	Development and differentiation enhancing factor 2	NM003887	-1.40	0.0228	-1.03
	LOC646082	Similar to Cysteine-rich protein 1	XM929042	-1.39	0.0159	-1.17
	LOC649571	Similar to Cysteine-rich protein 1	XM938642	-1.39	0.0159	-1.17
	MAFF	Musculoaponeurotic fibrosarcoma F, variants: 1&2	NM:012323;152878	-1.39	0.0463	-1.21
Pattern IV	VPS54	Vacuolar protein sorting 54, variants: 1&2	NM:016516;001005739	-1.33	0.0159	-1.11
	STBD1	Starch binding domain 1	NM003943	-1.32	0.0005	-1.11
	TAPT1	Transmembrane anterior posterior transformation 1	NM153365	-1.30	0.0171	-1.10
	SVIP	Small VCP/p97-interacting protein, variants: 2&5	XM:934479;945595	-1.25	0.0197	-1.07
	MARK1	MAP/microtubule affinity-regulating kinase 1	NM018650	-1.23	0.0085	-1.00
	GPRIN2	G protein regulated inducer of neurite outgrowth 2	NM014696	-1.23	0.0465	-1.01
	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	NM015326;XM936300	-1.22	0.0023	-1.02

[a] -Fold change: Ad-GFP + EtOH *versus* Ad-GFP + DHT.
[b] -Fold change: Ad-GFP + DHT *versus* Ad-D1 + DHT.

all 393 androgen-regulated and cyclin D1-sensitive transcripts (using the DAVID Bioinformatics Resource) revealed significant associations with diverse biological processes (supplemental Table 1), many of which are expected, based on known functions of cyclin D1 (*i.e.* regulation of cell cycle). Gene ontology analysis of the individual patterns was limited by the small number of transcripts identified in Patterns III and IV (supplemental Fig. 4), thus confounding the ability to assess the overall impact of cyclin D1 on androgen signaling. Therefore, the top regulated transcripts for each pattern were assessed to identify potential commonality (Table 1). Interestingly, among the top androgen-regulated transcripts sensitive to cyclin D1, Pattern I contained a number of known AR target genes (*i.e. KLK* genes), consistent with the role of cyclin D1 in negatively regulating *KLK3/PSA* expression (29).

Cyclin D1 Antagonizes Androgen-dependent Up-regulation of AR Target Genes—Because the data above suggest that cyclin D1 may selectively inhibit AR function, it became necessary to validate these findings through quantitative assessment of other known and putative AR target genes (Fig. 3*A*). As an addi-

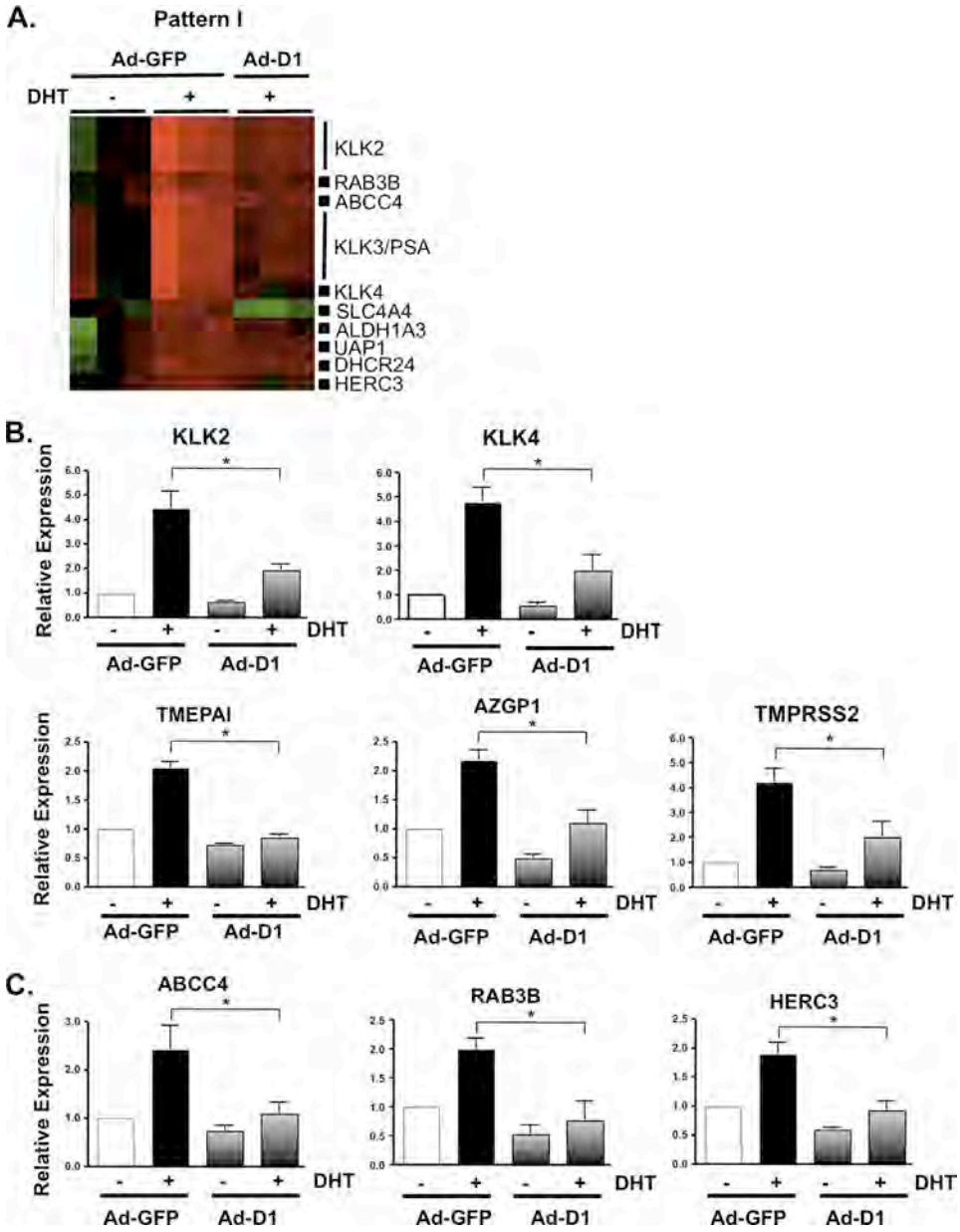

FIGURE 3. **Cyclin D1 attenuates AR-dependent gene expression.** *A*, to determine the role of cyclin D1 on AR-dependent gene expression, heat map analysis was performed on selected Pattern I transcripts that are frequently observed as androgen/AR-regulated transcripts. The relative expression of known AR target genes *KLK2*, *KLK4*, *TMEPAI*, *AZGP1*, and *TMPRSS2* (*B*) and putative AR target genes *ABCC4*, *RAB3B*, and *HERC3* (*C*) was performed by SYBR-based qPCR from three or four independent experiments and presented as described in the legend to Fig. 1. Cyclin D1-transduced cells treated with ethanol were included in the validation to assess the impact of cyclin D1 on basal transcription. All transcripts tested were validated as androgen-dependent; however, only the transcripts that demonstrated a significant ($p < 0.05$) difference in the presence of cyclin D1 are indicated by an *asterisk*. *D*, knockdown of cyclin D1, in LNCaP cells cultured under standard growth conditions (*i.e.* 5% FBS), was performed to further validate the influence of cyclin D1 on AR target gene expression. A representative immunoblot for cyclin D1 knockdown is provided. The relative expression of *KLK3/PSA*, *TMPRSS2*, and *ABCC4* was determined by qPCR, from three individual experiments, and plotted as described above. *E*, the transcripts from Pattern I were assessed, bioinformatically, for ARORs within 50 kb of the TSS using a publicly available data set, as described under "Experimental Procedures," to determine the overall potential of AR to regulate these gene loci.

tional control, expression of these transcripts was measured in non-DHT-stimulated cells to determine the relevance of cyclin D1-mediated repression on basal AR activity. Consistent with observations for *KLK3/PSA* expression (Fig. 1*B*), cyclin D1 repressed the androgen-dependent induction but not basal expression of the other kallikrein family members *KLK2* and *KLK4* by 56.8 and 58.7%, respectively (Fig. 3*B*). The kallikrein genes are located in the same genomic cluster on chromosome 19 (53); to determine whether the effects of cyclin D1 on known AR target genes were specific to this chromosomal location, the impact of cyclin D1 status on expression of established AR target genes residing on distinct loci (*TMEPAI*, *AZGP1*, and *TMPRSS2*; chromosomes 20 (54), 7 (55, 56), and 21 (57), respectively) was determined. Each was significantly induced by androgen, consistent with previous studies (56, 58–61). Furthermore, ligand-induced gene expression was reduced in the presence of cyclin D1 (58.5, 49.5, and 52%, respectively). Together, these data indicate that the repressive capacity of cyclin D1 on androgen-induced expression of known AR target genes is not specific to one gene cluster and/or chromosomal position.

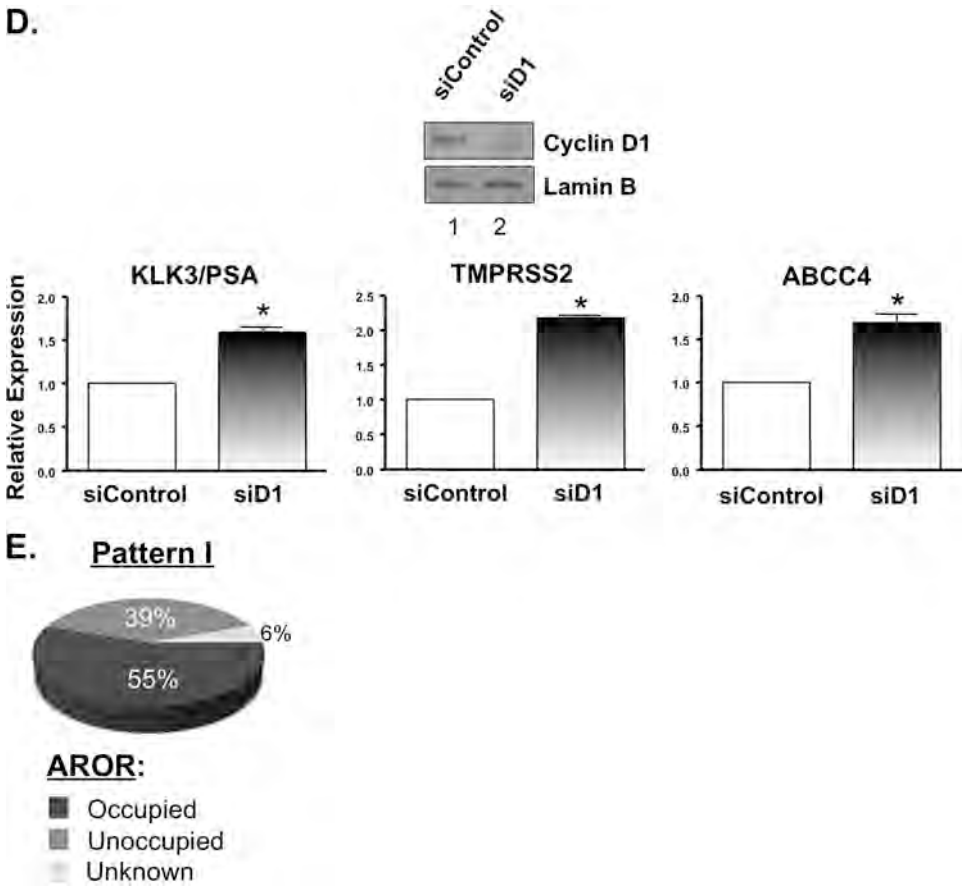

FIGURE 3—*continued*

Last, quantitative analyses were performed for genes induced by androgen and suspected to be directly regulated by AR (62). Importantly, each gene has been recently shown to have potential AR binding sites within regions capable of altering gene expression (40) and may have significance in PCa. *ABCC4* (which encodes a ATP-binding cassette transporter) expression is increased in PCa (63, 64) and, as a multidrug resistance protein (MRP) family member, may contribute to drug resistance (65). *RAB3B* (which encodes a vesicular transport protein of the RAS oncogene family) and *HERC3* (which encodes a HECT domain E3 ubiquitin-protein ligase) were also investigated. As shown in Fig. 3*C*, androgen-induced expression of *ABCC4*, *RAB3B*, and *HERC3* was diminished by cyclin D1 reconstitution (54.6, 61.6, and 51.2%, respectively). Analysis of four other androgen/AR-dependent transcripts identified in Pattern I (*DHCR24*, *ALDH1A3*, *UAP1*, and *SLC4A4*) revealed a consistent trend for cyclin D1-mediated suppression of androgen-dependent expression (data not shown). Importantly, expression of *HERC3* and *ABCC4* (both of which are induced by androgen in the VCaP model) was significantly inhibited by cyclin D1 (supplemental Fig. 5*A*). Interestingly, only weak induction of *RAB3B* was observed upon androgen treatment in the VCaP cells (data not shown), indicating potential differences in AR function between model systems. Conversely, siRNA-mediated ablation of endogenous cyclin D1 in LNCaP cells was sufficient to deregulate AR target gene expression (Fig. 3*D*), further indicating the importance of cyclin D1 in modulating AR function.

A crucial step in AR-dependent transcription is recruitment of the receptor to chromatin, and a recent study revealed that the majority of ARORs are located within 50 kb of the TSS of a gene (40). These data prompted a bioinformatic evaluation of the Pattern I transcripts to further define which loci of the cyclin D1-sensitive transcripts have ARORs near the TSS. Interestingly, more than half (55%) of Pattern I contained ARORs within 50 kb of the TSS (Fig. 3*E*), suggesting that the majority of these cyclin D1-sensitive transcripts are probably regulated by AR in a direct fashion. Combined, these analyses not only identify a gene-selective cyclin D1-responsive signature but also demonstrate that cyclin D1 significantly attenuates ligand-induced gene expression of AR target genes with potential PCa importance.

AR Residence on Chromatin Is Regulated by Cyclin D1—To further explore the mechanisms by which cyclin D1 specifically acts to suppress AR target gene expression, ChIP assays were performed. Despite the increasingly large number of potential androgen-regulated genes with ARORs (40, 66–69), only a few genes have been validated for functional output due to AR binding (70). The *KLK3/PSA* regulatory locus was initially analyzed (Fig. 4*A*), wherein AR recruitment to the *KLK3/PSA* enhancer region has been documented to occur with an initial periodicity of 60–90 min (71–74). Consistent with these findings, DHT induced AR recruitment after 1 h, and AR occupancy was maintained at the 3 h time point (5.8- and 5.9-fold over vehicle control, respectively). Importantly, restoration of cyclin D1 reduced androgen-induced AR occupancy at both time points

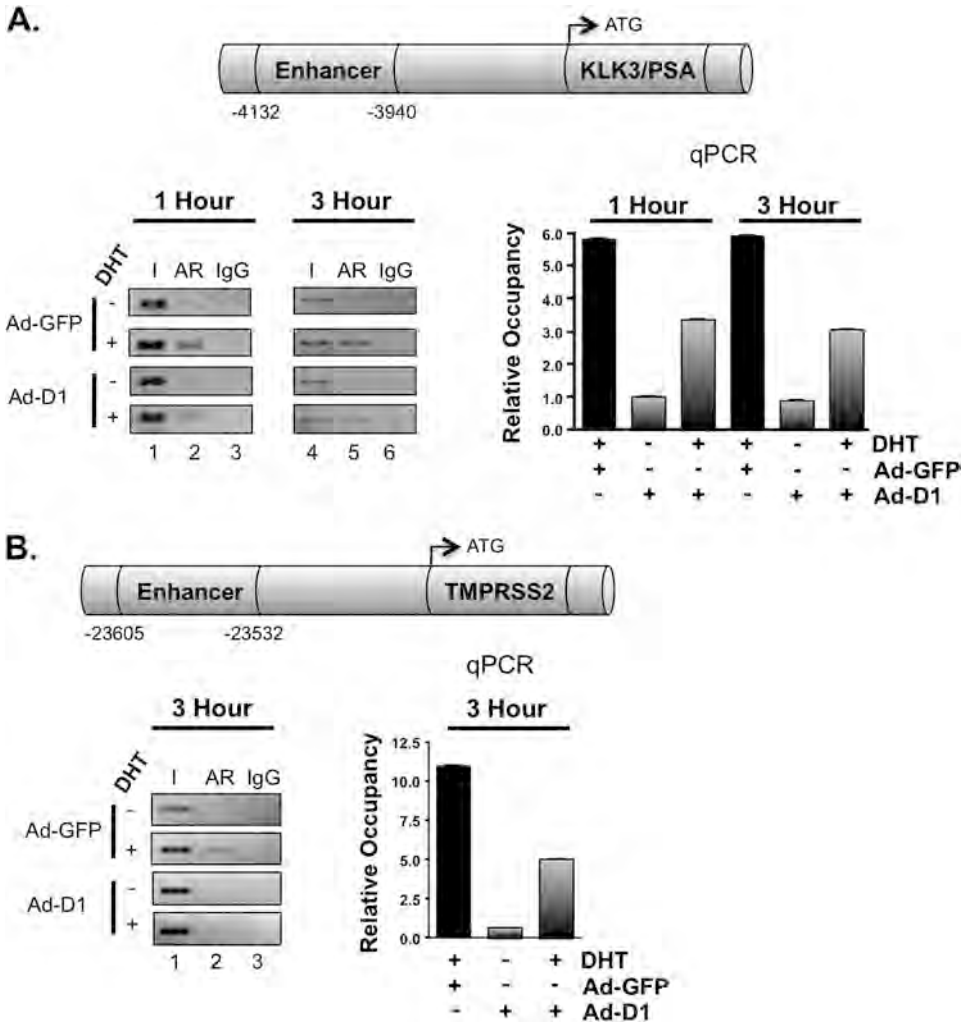

FIGURE 4. **Cyclin D1 displaces AR occupancy at target gene loci.** ChIP analysis was performed to determine the influence of cyclin D1 on AR occupancy. LNCaP cells were treated as described in the legend to Fig. 2, except cells were stimulated with 10 nM DHT for 1–3 h. *Bar graphs* represent the relative occupancy ± S.D. (*error bars*) from a representative AR ChIP, where each condition is a biological triplicate. *A*, *schematic*, *KLK3/PSA* locus showing the location of the well characterized, AR-responsive enhancer region upstream of the TSS. *Bar graph*, qPCR analysis for the enhancer region from AR ChIP assays at 1 and 3 h. Representative conventional PCR is provided (*left*). *B*, *schematic*, *TMPRSS2* locus showing the location of the AR-responsive enhancer region. *Bar graph*, qPCR analysis for the enhancer region from an AR ChIP assay at 3 h. Representative conventional PCR is provided (*left*).

(42.4 and 48.5%, respectively). These data are consistent with the magnitude of *KLK3/PSA* expression changes observed by qPCR analysis (Fig. 1*B*) and microarray expression profiling (Fig. 3*A*). Similar results were observed in the VCaP model (supplemental Fig. 5*B*). However, a more modest reduction in AR occupancy was observed after cyclin D1 transduction, as might be expected because VCaP cells harbor amplification of the AR locus and express higher levels of the receptor (75, 76). Together, these data yielded the first indication that cyclin D1 alters AR association with chromatin. To assess these findings further, the impact of cyclin D1 on AR occupancy was performed using the *TMPRSS2* locus (Fig. 4*B*), whose AR-dependent regulatory region has been recently identified in a chromosomal translocation event of high significance in PCa (77). Of the five potential AREs associated with the *TMPRSS2* locus, the enhancer region (ARE V) is the predominant site regulating androgen responsiveness and AR recruitment (45). Similar to observations at the *KLK3/PSA* locus, cyclin D1 suppressed DHT-stimulated AR occupancy at the *TMPRSS2* enhancer by 54.3%. Together, these data demonstrate that a predominant transcriptional consequence of cyclin D1 with regard to AR target gene regulation is to suppress DHT-induced AR occupancy and target gene expression and provide new insight into the potential consequences of aberrant cyclin D1 expression in human disease.

DISCUSSION

It is now apparent that a major function of cyclin D1 *in vivo* is to bind and regulate transcription factor action. Findings supporting this contention are robust, and multiple studies have validated the importance of cyclin D1-mediated transcriptional regulation with regard to cellular and *in vivo* outcomes (15, 16). In PCa, previous studies established a paradigm whereby cyclin D1 attenuates AR-mediated *KLK3/PSA* expression and established that this ability to suppress AR function is subverted in human disease through multiple mechanisms (29). Despite these advances, previous reports have been limited to a small subset of AR target genes, and the overall consequence of cyclin

D1 on androgen-dependent gene expression has remained elusive. Here, an unbiased approach was used to illuminate three critical facets of cyclin D1 function. First, it was observed that cyclin D1 selectively regulates androgen-dependent programming. Unexpectedly, cyclin D1 was able to oppose and enhance androgen function in a gene-selective manner, thus providing a new understanding of how altered cyclin D1 expression and/or function can rewire the cellular response to androgen stimulation. Second, it was shown that androgen-induced transcripts that are direct AR target genes were suppressed by cyclin D1, thus providing unbiased evidence that a primary function of cyclin D1 is to limit AR activity induced by ligand. Third, it was demonstrated that cyclin D1 markedly reduced AR residence on clinically relevant gene loci, thus identifying a new mechanism of cyclin D1 action. Taken together, these studies identify the transcriptional regulatory functions of cyclin D1 as critical effectors of androgen-dependent signaling and AR-associated chromatin dynamics.

The capacity of cyclin D1 to coordinate mitogenic signals through the G_1-S cell cycle machinery is exceedingly well understood and is frequently cited as an important driver of tumorigenesis (3). However, cyclin D1 also interacts with and modulates multiple transcription factors, including prominent members of the nuclear hormone receptor superfamily: estrogen receptor α (20), thyroid hormone receptor (21), peroxisome proliferator-activated receptor γ (33), and AR (29). Little is known concerning the overall impact of cyclin D1 on the transcriptional regulatory networks of these nuclear receptors. The prostate is a unique model to study the transcriptional consequence of cyclin D1 because androgens are important for the growth and survival of PCa cells. The mechanisms by which cyclin D1 elicits transcriptional repression have been preliminarily characterized (23, 30, 32, 34, 36), and the clinical importance has been suggested because human PCa specimens that lack cyclin D1 are associated with elevated serum PSA (27). The current study was conducted using physiological concentrations of androgen, and the number of overall androgen-regulated transcripts identified is consistent with previous reports (78–81). The present study is one of the first to determine the global impact of cyclin D1 on hormone-dependent gene expression, and the transcriptional patterns identified probably impact a broad range of cellular processes, especially cell cycle control and metabolism, both of which are consistent with the ability androgens to influence growth and differentiation. Further studies will be required to understand the complex role cyclin D1 plays with regard to these biological functions. Clearly, these observations indicate that cyclin D1 regulates a complex, androgen-dependent gene expression profile.

The finding that cyclin D1 can both antagonize and synergize with androgen-regulated gene programming was unexpected. As shown, *k*-means analyses clustered an overwhelming majority of putative and known AR target genes, including *KLK3/PSA*, into Pattern I (androgen-induced, cyclin D1-repressed). Many of the AR target genes that were validated in the current study are involved in processes such as catabolism (*i.e. KLK* genes, *TMEPAI*, *AZGP1*, and *HERC3*) or transport (*i.e. ABCC4* and *RAB3B*), consistent with the ability of androgens to regulate growth and metabolic phenotypes, as was recently indicated through combined gene expression and proteomic profiling (82). Genome-wide association data in LNCaP cells suggested that AR binding is enriched at androgen-activated but not androgen-repressed genes (40). Consistent with this notion, overlay of genome-wide data with Pattern I genes demonstrated that AR occupancy was enriched in this subset of androgen-induced transcripts. Currently, it remains to be determined if other potential AR-regulated genes within Pattern I also contribute to the growth and differentiation phenotype. However, with regard to those androgen-induced and AR-mediated transcriptional events, these data are consistent with the model that cyclin D1 negatively regulates AR function.

In contrast, Pattern II (androgen-induced and cyclin D1-induced) contained a paucity of known or putative AR target genes, suggesting that the observed synergy between cyclin D1 and androgen is probably the result of secondary transcriptional effects that may include CDK-dependent functions. Consonantly, many Pattern II transcripts are also regulated by the E2F family of transcription factors, and similar results were observed in previous studies using the murine liver, wherein deregulation of cyclin D1 resulted in transcriptional changes in known E2F genes, including *CDC6*, *CDT1*, *RRM2*, *MCM2*, *MCM4*, and *MCM5* (83). Alternatively, $p21^{Cip1}$ can facilitate the assembly of cyclin D1 with CDKs and regulate their subsequent nuclear localization (84–86), and it has been shown that $p21^{Cip1}$ interacts with estrogen receptor α and behaves as a transcriptional co-activator in a gene-specific manner (87). Thus, $p21^{Cip1}$ may serve to facilitate the simultaneous androgen and cyclin D1-mediated activation of Pattern II genes, which could potentially explain the perplexing PCa clinical data indicating that increased $p21^{Cip1}$ is associated with decreased survival (88–91). Overall, these findings demonstrate that cyclin D1 is a selective modifier of androgen-induced gene expression, wherein cyclin D1 utilizes disparate mechanisms to potentiate a subset of androgen-induced genes but preferentially suppresses genes directly regulated by AR.

The finding that cyclin D1 alters AR residence on chromatin of well characterized AR target genes within Pattern I suggests that a mechanism of cyclin D1-mediated transcriptional control occurs through altering transcription factor-chromatin interactions. Notably, cyclin D1 is known to suppress androgen-induced N-C-terminal interaction in the AR (30), which has been hypothesized to facilitate chromatin binding (92). Similarly, the ability of cyclin D1 to inhibit *KLK3/PSA* gene expression through histone deacetylase involvement (32, 34) suggests that alterations in the chromatin microenvironment probably contribute to the observed effects within Pattern I. Currently, only a few genes have been extensively characterized with regard to AR binding, and future directions will challenge the concept that cyclin D1 influences the association of AR with chromatin. Moreover, in keeping with the notion that cyclin D1 alters transcription factor-chromatin interactions, recent analyses demonstrated that cyclin D1 serves a significant transcriptional role during mouse retinal development (15). Thus, it will be of future interest to utilize genome-wide methods to characterize the extent of cyclin D1 chromatin association in the prostate.

In summary, cyclin D1 has been shown previously to be aberrantly regulated in PCa (27). In the current study, unbiased gene expression profiling illuminated new and unexpected functions for cyclin D1 in this tissue type. These data identify a "signature" of cyclin D1 activity that impinges on androgen-dependent signaling and demonstrate that AR target genes of clinical relevance are suppressed by cyclin D1. Further assessment of cyclin D1 function revealed a potential new mechanism of action, wherein cyclin D1 limits AR occupancy at endogenous loci. However, a subset of androgen-induced genes were potentiated by cyclin D1, thus demonstrating that cyclin D1 exerts complex, pleiotropic effects on hormone action. Together, these data identify cyclin D1 as a selective effector of androgen-dependent signaling and AR-associated chromatin dynamics.

Acknowledgments—We thank the members of the Knudsen lab and the group of E. Knudsen for insightful discussions and critical reading of the manuscript.

REFERENCES

1. Ewen, M. E., and Lamb, J. (2004) *Trends Mol. Med.* **10,** 158–162
2. Fu, M., Wang, C., Li, Z., Sakamaki, T., and Pestell, R. G. (2004) *Endocrinology* **145,** 5439–5447
3. Kim, J. K., and Diehl, J. A. (2009) *J. Cell. Physiol.* **220,** 292–296
4. Knudsen, K. E. (2006) *Cell Div.* **1,** 15
5. Bates, S., Bonetta, L., MacAllan, D., Parry, D., Holder, A., Dickson, C., and Peters, G. (1994) *Oncogene* **9,** 71–79
6. Matsushime, H., Ewen, M. E., Strom, D. K., Kato, J. Y., Hanks, S. K., Roussel, M. F., and Sherr, C. J. (1992) *Cell* **71,** 323–334
7. Meyerson, M., and Harlow, E. (1994) *Mol. Cell. Biol.* **14,** 2077–2086
8. Yu, Q., Ciemerych, M. A., and Sicinski, P. (2005) *Oncogene* **24,** 7114–7119
9. Ciemerych, M. A., Yu, Q., Szczepanska, K., and Sicinski, P. (2008) *Int. J. Dev. Biol.* **52,** 299–305
10. Bernards, R. (1999) *Biochim. Biophys. Acta* **1424,** M17–22
11. Coqueret, O. (2002) *Gene* **299,** 35–55
12. Kozar, K., Ciemerych, M. A., Rebel, V. I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R. T., Akashi, K., and Sicinski, P. (2004) *Cell* **118,** 477–491
13. Malumbres, M., Sotillo, R., Santamaría, D., Galán, J., Cerezo, A., Ortega, S., Dubus, P., and Barbacid, M. (2004) *Cell* **118,** 493–504
14. Landis, M. W., Pawlyk, B. S., Li, T., Sicinski, P., and Hinds, P. W. (2006) *Cancer Cell* **9,** 13–22
15. Bienvenu, F., Jirawatnotai, S., Elias, J. E., Meyer, C. A., Mizeracka, K., Marson, A., Frampton, G. M., Cole, M. F., Odom, D. T., Odajima, J., Geng, Y., Zagozdzon, A., Jecrois, M., Young, R. A., Liu, X. S., Cepko, C. L., Gygi, S. P., and Sicinski, P. (2010) *Nature* **463,** 374–378
16. Lamb, J., Ramaswamy, S., Ford, H. L., Contreras, B., Martinez, R. V., Kittrell, F. S., Zahnow, C. A., Patterson, N., Golub, T. R., and Ewen, M. E. (2003) *Cell* **114,** 323–334
17. Gan, L., Liu, P., Lu, H., Chen, S., Yang, J., McCarthy, J. B., Knudsen, K. E., and Huang, H. (2009) *Cell Death Differ.* **16,** 1408–1417
18. Inoue, K., and Sherr, C. J. (1998) *Mol. Cell. Biol.* **18,** 1590–1600
19. Neuman, E., Ladha, M. H., Lin, N., Upton, T. M., Miller, S. J., DiRenzo, J., Pestell, R. G., Hinds, P. W., Dowdy, S. F., Brown, M., and Ewen, M. E. (1997) *Mol. Cell. Biol.* **17,** 5338–5347
20. Zwijsen, R. M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R. J. (1997) *Cell* **88,** 405–415
21. Lin, H. M., Zhao, L., and Cheng, S. Y. (2002) *J. Biol. Chem.* **277,** 28733–28741
22. Wang, C., Pattabiraman, N., Zhou, J. N., Fu, M., Sakamaki, T., Albanese, C., Li, Z., Wu, K., Hulit, J., Neumeister, P., Novikoff, P. M., Brownlee, M., Scherer, P. E., Jones, J. G., Whitney, K. D., Donehower, L. A., Harris, E. L., Rohan, T., Johns, D. C., and Pestell, R. G. (2003) *Mol. Cell. Biol.* **23,** 6159–6173
23. Knudsen, K. E., Cavenee, W. K., and Arden, K. C. (1999) *Cancer Res.* **59,** 2297–2301
24. Reutens, A. T., Fu, M., Wang, C., Albanese, C., McPhaul, M. J., Sun, Z., Balk, S. P., Jänne, O. A., Palvimo, J. J., and Pestell, R. G. (2001) *Mol. Endocrinol.* **15,** 797–811
25. Burd, C. J., Petre, C. E., Morey, L. M., Wang, Y., Revelo, M. P., Haiman, C. A., Lu, S., Fenoglio-Preiser, C. M., Li, J., Knudsen, E. S., Wong, J., and Knudsen, K. E. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 2190–2195
26. Comstock, C. E., Augello, M. A., Benito, R. P., Karch, J., Tran, T. H., Utama, F. E., Tindall, E. A., Wang, Y., Burd, C. J., Groh, E. M., Hoang, H. N., Giles, G. G., Severi, G., Hayes, V. M., Henderson, B. E., Le Marchand, L., Kolonel, L. N., Haiman, C. A., Baffa, R., Gomella, L. G., Knudsen, E. S., Rui, H., Henshall, S. M., Sutherland, R. L., and Knudsen, K. E. (2009) *Clin Cancer Res.* **15,** 5338–5349
27. Comstock, C. E., Revelo, M. P., Buncher, C. R., and Knudsen, K. E. (2007) *Br. J. Cancer* **96,** 970–979
28. Xu, Y., Chen, S. Y., Ross, K. N., and Balk, S. P. (2006) *Cancer Res.* **66,** 7783–7792
29. Burd, C. J., Morey, L. M., and Knudsen, K. E. (2006) *Endocr. Relat. Cancer* **13,** 979–994
30. Burd, C. J., Petre, C. E., Moghadam, H., Wilson, E. M., and Knudsen, K. E. (2005) *Mol. Endocrinol.* **19,** 607–620
31. Li, J., Lin, Q., Wang, W., Wade, P., and Wong, J. (2002) *Genes Dev.* **16,** 687–692
32. Petre-Draviam, C. E., Williams, E. B., Burd, C. J., Gladden, A., Moghadam, H., Meller, J., Diehl, J. A., and Knudsen, K. E. (2005) *Oncogene* **24,** 431–444
33. Fu, M., Rao, M., Bouras, T., Wang, C., Wu, K., Zhang, X., Li, Z., Yao, T. P., and Pestell, R. G. (2005) *J. Biol. Chem.* **280,** 16934–16941
34. Petre, C. E., Wetherill, Y. B., Danielsen, M., and Knudsen, K. E. (2002) *J. Biol. Chem.* **277,** 2207–2215
35. Knudsen, K. E., and Scher, H. I. (2009) *Clin. Cancer Res.* **15,** 4792–4798
36. Schiewer, M. J., Morey, L. M., Burd, C. J., Liu, Y., Merry, D. E., Ho, S. M., and Knudsen, K. E. (2009) *Oncogene* **28,** 1016–1027
37. Knudsen, K. E., Arden, K. C., and Cavenee, W. K. (1998) *J. Biol. Chem.* **273,** 20213–20222
38. de Launoit, Y., Veilleux, R., Dufour, M., Simard, J., and Labrie, F. (1991) *Cancer Res.* **51,** 5165–5170
39. Dai, M., Wang, P., Boyd, A. D., Kostov, G., Athey, B., Jones, E. G., Bunney, W. E., Myers, R. M., Speed, T. P., Akil, H., Watson, S. J., and Meng, F. (2005) *Nucleic Acids Res.* **33,** e175
40. Wang, Q., Li, W., Zhang, Y., Yuan, X., Xu, K., Yu, J., Chen, Z., Beroukhim, R., Wang, H., Lupien, M., Wu, T., Regan, M. M., Meyer, C. A., Carroll, J. S., Manrai, A. K., Jänne, O. A., Balk, S. P., Mehra, R., Han, B., Chinnaiyan, A. M., Rubin, M. A., True, L., Fiorentino, M., Fiore, C., Loda, M., Kantoff, P. W., Liu, X. S., and Brown, M. (2009) *Cell* **138,** 245–256
41. Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., and Haussler, D. (2002) *Genome Res.* **12,** 996–1006
42. Bussey, K. J., Kane, D., Sunshine, M., Narasimhan, S., Nishizuka, S., Reinhold, W. C., Zeeberg, B., Ajay, W., and Weinstein, J. N. (2003) *Genome Biol.* **4,** R27
43. Wetherill, Y. B., Petre, C. E., Monk, K. R., Puga, A., and Knudsen, K. E. (2002) *Mol. Cancer Ther.* **1,** 515–524
44. Link, K. A., Balasubramaniam, S., Sharma, A., Comstock, C. E., Godoy-Tundidor, S., Powers, N., Cao, K. H., Haelens, A., Claessens, F., Revelo, M. P., and Knudsen, K. E. (2008) *Cancer Res.* **68,** 4551–4558
45. Wang, Q., Li, W., Liu, X. S., Carroll, J. S., Jänne, O. A., Keeton, E. K., Chinnaiyan, A. M., Pienta, K. J., and Brown, M. (2007) *Mol. Cell* **27,** 380–392
46. Henrique, R., Costa, V. L., Cerveira, N., Carvalho, A. L., Hoque, M. O., Ribeiro, F. R., Oliveira, J., Teixeira, M. R., Sidransky, D., and Jerónimo, C. (2006) *J. Mol. Med.* **84,** 911–918
47. Müller, I., Wischnewski, F., Pantel, K., and Schwarzenbach, H. (2010) *BMC Cancer* **10,** 297
48. Peng, Y., Chen, F., Melamed, J., Chiriboga, L., Wei, J., Kong, X., McLeod, M., Li, Y., Li, C. X., Feng, A., Garabedian, M. J., Wang, Z., Roeder, R. G., and Lee, P. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 5236–5241
49. Wissmann, C., Wild, P. J., Kaiser, S., Roepcke, S., Stoehr, R., Woenckhaus, M., Kristiansen, G., Hsieh, J. C., Hofstaedter, F., Hartmann, A., Knuechel,

R., Rosenthal, A., and Pilarsky, C. (2003) *J. Pathol.* **201,** 204–212
50. Olshavsky, N. A., Groh, E. M., Comstock, C. E., Morey, L. M., Wang, Y., Revelo, M. P., Burd, C., Meller, J., and Knudsen, K. E. (2008) *Oncogene* **27,** 3111–3121
51. Lam, E. W., Glassford, J., Banerji, L., Thomas, N. S., Sicinski, P., and Klaus, G. G. (2000) *J. Biol. Chem.* **275,** 3479–3484
52. Petre-Draviam, C. E., Cook, S. L., Burd, C. J., Marshall, T. W., Wetherill, Y. B., and Knudsen, K. E. (2003) *Cancer Res.* **63,** 4903–4913
53. Obiezu, C. V., and Diamandis, E. P. (2005) *Cancer Lett.* **224,** 1–22
54. Xu, L. L., Shanmugam, N., Segawa, T., Sesterhenn, I. A., McLeod, D. G., Moul, J. W., and Srivastava, S. (2000) *Genomics* **66,** 257–263
55. Pendás, A. M., Matilla, T., Uría, J. A., Freije, J. P., Fueyo, A., Estivill, X., and López-Otín, C. (1994) *Cytogenet. Cell Genet.* **66,** 263–266
56. Nelson, P. S., Clegg, N., Arnold, H., Ferguson, C., Bonham, M., White, J., Hood, L., and Lin, B. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 11890–11895
57. Lin, B., Ferguson, C., White, J. T., Wang, S., Vessella, R., True, L. D., Hood, L., and Nelson, P. S. (1999) *Cancer Res.* **59,** 4180–4184
58. Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. (2004) *Nat. Med.* **10,** 33–39
59. DePrimo, S. E., Diehn, M., Nelson, J. B., Reiter, R. E., Matese, J., Fero, M., Tibshirani, R., Brown, P. O., and Brooks, J. D. (2002) *Genome Biol.* **3,** RESEARCH0032
60. Holzbeierlein, J., Lal, P., LaTulippe, E., Smith, A., Satagopan, J., Zhang, L., Ryan, C., Smith, S., Scher, H., Scardino, P., Reuter, V., and Gerald, W. L. (2004) *Am J. Pathol.* **164,** 217–227
61. Zhao, H., Kim, Y., Wang, P., Lapointe, J., Tibshirani, R., Pollack, J. R., and Brooks, J. D. (2005) *Prostate* **63,** 187–197
62. Comstock, C. E., Burd, C. J., Jessen, W. J., and Knudsen, K. E. (2008) in *Contemporary Endocrinology: Genomics in Endocrinology-DNA Microarray Analysis in Endocrine Health and Disease* (Handwerger, S., and Aronow, B. J., eds) pp. 83–113, Humana Press, New Jersey
63. Cai, C., Omwancha, J., Hsieh, C. L., and Shemshedini, L. (2007) *Prostate Cancer Prostatic Dis.* **10,** 39–45
64. Ho, L. L., Kench, J. G., Handelsman, D. J., Scheffer, G. L., Stricker, P. D., Grygiel, J. G., Sutherland, R. L., Henshall, S. M., Allen, J. D., and Horvath, L. G. (2008) *Prostate* **68,** 1421–1429
65. Kruh, G. D., Belinsky, M. G., Gallo, J. M., and Lee, K. (2007) *Cancer Metastasis Rev.* **26,** 5–14
66. Jia, L., Berman, B. P., Jariwala, U., Yan, X., Cogan, J. P., Walters, A., Chen, T., Buchanan, G., Frenkel, B., and Coetzee, G. A. (2008) *PLoS One* **3,** e3645
67. Lin, B., Wang, J., Hong, X., Yan, X., Hwang, D., Cho, J. H., Yi, D., Utleg, A. G., Fang, X., Schones, D. E., Zhao, K., Omenn, G. S., and Hood, L. (2009) *PLoS One* **4,** e6589
68. Massie, C. E., Adryan, B., Barbosa-Morais, N. L., Lynch, A. G., Tran, M. G., Neal, D. E., and Mills, I. G. (2007) *EMBO Rep.* **8,** 871–878
69. Bolton, E. C., So, A. Y., Chaivorapol, C., Haqq, C. M., Li, H., and Yamamoto, K. R. (2007) *Genes Dev.* **21,** 2005–2017
70. Makkonen, H., Kauhanen, M., Paakinaho, V., Jääskeläinen, T., and Palvimo, J. J. (2009) *Nucleic Acids Res.* **37,** 4135–4148
71. Clark, E. L., Coulson, A., Dalgliesh, C., Rajan, P., Nicol, S. M., Fleming, S., Heer, R., Gaughan, L., Leung, H. Y., Elliott, D. J., Fuller-Pace, F. V., and Robson, C. N. (2008) *Cancer Res.* **68,** 7938–7946
72. Shi, X. B., Xue, L., Zou, J. X., Gandour-Edwards, R., Chen, H., and deVere White, R. W. (2008) *Prostate* **68,** 1816–1826
73. Kang, Z., Jänne, O. A., and Palvimo, J. J. (2004) *Mol. Endocrinol.* **18,** 2633–2648
74. Kang, Z., Pirskanen, A., Jänne, O. A., and Palvimo, J. J. (2002) *J. Biol. Chem.* **277,** 48366–48371
75. Makkonen, H., Kauhanen, M., Jääskeläinen, T., and Palvimo, J. J. (2011) *Mol. Cell. Endocrinol.* **331,** 57–65
76. Waltering, K. K., Helenius, M. A., Sahu, B., Manni, V., Linja, M. J., Jänne, O. A., and Visakorpi, T. (2009) *Cancer Res.* **69,** 8141–8149
77. Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X. W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J. E., Shah, R. B., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. (2005) *Science* **310,** 644–648
78. Li, H., Lovci, M. T., Kwon, Y. S., Rosenfeld, M. G., Fu, X. D., and Yeo, G. W. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 20179–20184
79. Mendiratta, P., Mostaghel, E., Guinney, J., Tewari, A. K., Porrello, A., Barry, W. T., Nelson, P. S., and Febbo, P. G. (2009) *J. Clin Oncol.* **27,** 2022–2029
80. Ngan, S., Stronach, E. A., Photiou, A., Waxman, J., Ali, S., and Buluwela, L. (2009) *Oncogene* **28,** 2051–2063
81. Wang, G., Jones, S. J., Marra, M. A., and Sadar, M. D. (2006) *Oncogene* **25,** 7311–7323
82. Vellaichamy, A., Dezso, Z., JeBailey, L., Chinnaiyan, A. M., Sreekumar, A., Nesvizhskii, A. I., Omenn, G. S., and Bugrim, A. (2010) *PLoS One* **5,** e10936
83. Mullany, L. K., White, P., Hanse, E. A., Nelsen, C. J., Goggin, M. M., Mullany, J. E., Anttila, C. K., Greenbaum, L. E., Kaestner, K. H., and Albrecht, J. H. (2008) *Cell Cycle* **7,** 2215–2224
84. Alt, J. R., Gladden, A. B., and Diehl, J. A. (2002) *J. Biol. Chem.* **277,** 8517–8523
85. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999) *EMBO J.* **18,** 1571–1583
86. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997) *Genes Dev.* **11,** 847–862
87. Fritah, A., Saucier, C., Mester, J., Redeuilh, G., and Sabbah, M. (2005) *Mol. Cell. Biol.* **25,** 2419–2430
88. Lacombe, L., Maillette, A., Meyer, F., Veilleux, C., Moore, L., and Fradet, Y. (2001) *Int. J. Cancer* **95,** 135–139
89. Omar, E. A., Behlouli, H., Chevalier, S., and Aprikian, A. G. (2001) *Prostate* **49,** 191–199
90. Rigaud, J., Tiguert, R., Decobert, M., Hovington, H., Latulippe, E., Laverdiere, J., Larue, H., Lacombe, L., and Fradet, Y. (2004) *Prostate* **58,** 269–276
91. Sarkar, F. H., Li, Y., Sakr, W. A., Grignon, D. J., Madan, S. S., Wood, D. P., Jr., and Adsay, V. (1999) *Prostate* **40,** 256–260
92. Wong, C. I., Zhou, Z. X., Sar, M., and Wilson, E. M. (1993) *J. Biol. Chem.* **268,** 19004–19012